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**Outside inside signalling in CD40-mediated B cell activation**

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**ABSTRACT:** CD40 is a member of the growing tumor necrosis factor receptor (TNF-R) family of molecules, and has been shown to play important roles in T cell-mediated B lymphocyte activation. Ligation of B cell CD40 by CD154 expressed on activated T cells stimulates B cell proliferation, differentiation, isotype switching, upregulation of surface molecules contributing to antigen presentation, development of the germinal center, and the humoral memory response. The present review will summarize recent literature data on the various CD40 signalling pathways, which involve both the TNF-R associated factors (TRAFs) and additional signalling proteins, and lead to activation of kinases and transcription factors.

**KEY WORDS:** CD40, B lymphocyte, Transcription factors, Signal transduction

**INTRODUCTION**

It has been recognized for over twenty years that signals in addition to those sent by the antigen receptor are required for full activation of a lymphocyte. Lymphocytes stimulated through the antigen receptor alone fail to produce cytokines, are unable to sustain proliferation, and often undergo apoptosis or become non-responsive to subsequent stimulation. CD40, the major B cell costimulatory molecule, was identified as a molecule expressed during all stages of B cell development and differentiation, whereas its ligand, CD40L (CD154, gp39, T-BAM, or TRAP), was mainly expressed on activated CD4\(^+\) T cells (1). Bearing the type I extracellular binding motif and further structural homologies, CD40 is considered a member of the TNF receptor superfamily, encompassing the TNF receptor type I (p55-TNFFR, CD120a), TNF receptor type II (p75-TNFFR, CD120b), low-affinity nerve growth factor receptor, CD27, CD30, CD95 (Fas/Apo), OX40, and 4-1BB (2). As typical for members of the TNF receptor superfamily, CD40 is characterized by a repetitive amino acid sequence pattern of four cysteine-enriched subdomains, typically consisting of six cysteines forming three disulfide domains. The intracellular domain of CD40, however, does not display a close relationship to other members of the family. The cytoplasmic domain of CD40 contains at least two major signalling determinants that include threonine 227 and 234 (3, 4). Human and murine CD40 share 62% homology at the amino acid level throughout their open reading frames (78% for the intracellular domain, 100% for the C-terminal 32 residues). Activation of the murine gene results in two mRNA species (1.4- and 1.7-kb form) by alternative usage of polyadenylation signals in the 3′ untranslated region, providing, however, identical coding sequences (5). Translation of the 1.5-kb CD40 mRNA generates an immunoreactive protein with a molecular weight of 43D50 kDa, mostly reported as a doublet consisting of a 43-kDa and a 47-kDa protein (6, 7). Furthermore, dimer formation has been described in B lymphocytes (6).

Embryogenic B lymphocytes already express...
CD40 (8), consistent with a functional role for CD40 in B cell ontogeny (9, 10). In addition to its role in early ontogeny, nearly every human adult B lymphocyte expresses CD40 regardless of its function (naïve cell, centroblast, plasmablast, plasma cell, memory cell) or location (bone marrow, tonsil, spleen, primary/secondary follicles) (11, 12). Furthermore, most malignant/leukemic B cell lines express CD40 independently of the degree of maturation of the affected lineage (8, 13-16). Apart from hematopoietic cells, the expression of CD40 has now been observed on many other cell lineages (Table I) (1). Extensive studies on CD40 activation of B cells in vitro have demonstrated that CD40 activation has major effects on many steps of the B cell natural history (12). CD40 activates proliferation, differentiation, and Ig production of immature and mature B cell subsets. In addition, CD40 can induce re-expression of telomerase activity in memory B cells, thereby contributing to an expanded lifespan of these cells (17). CD40 activation guides the B cells through their differentiation program, including rescue from apoptosis, differentiation into germinal center cells, isotype switching, selection, and maturation into memory cells. However, CD40 prevents the terminal differentiation of activated mature B cells into plasma cells (18, 19).

In vitro studies have shown direct effects of CD40 activation on cytokine production (IL-6, IL-10, TNF-α, LT-α), expression of adhesion molecules and costimulatory receptors (ICAM, CD23, B7.1/CD80, B7.2/CD86), and increased expression of MHC class I, MHC class II, and TAP transporter by B lymphocytes (20). For most processes, CD40 acts in concert with either cytokines or other receptor-ligand interactions. An important example of such cooperation is the process of isotype switching, which is initiated by CD40, but for which the specificity of the isotype is determined by cytokines. In humans, IL-4/IL-13 induce the switch to IgE and IgG4, which is fine-tuned by other cytokines. IL-7 (via the induction of IL-9 and sCD23) might enhance class switching to IgE and IgG4 (21), whereas addition of IL-10 inhibits IgE, but promotes IgG4 production (22). Switching to IgG1 and IgG3 is induced by IL-10, whereas IgG2 is induced by an as yet unidentified T cell factor. Finally, the switch to IgA production is promoted by a combination of IL-10 plus transforming growth factor β (TGF-β). More recently, it has become clear that other molecular interactions, like interaction with dendritic cell (DC), also might enhance the level of Ig production (23), or even directly promote the switch to a specific isotype (IgA) (24).

CD40 signalling pathway

Despite the impressive amount of data garnered within recent years, our knowledge regarding CD40-mediated signal transduction pathways and associated transducers remains incomplete and controversial. The controversies result, in part, from differences in CD40-mediated signal transduction among cell types and, furthermore, can vary within the same cell type depending on the stage of differentiation (25). Based on the structural homologies with the TNF/TNF receptor family, CD40 has been predicted to form trimeric structures, which as a consequence of receptor-ligand interaction results in the trimerization of CD40 receptor proteins (26). Indeed, CD154 and subsequent CD40 oligomerization are crucial steps in CD40-mediated signal transduction; hence, trimeric CD154 molecules exhibit the higher potency compared to monomeric or dimeric forms (27).

Although CD40 has no kinase domain, CD40 ligation activates several second messenger systems (Fig. 1). Among the earliest detectable events after CD40 activation are the activation of protein tyrosine kinases (PTK; including lyn, syk, and Jak3), activation of PI3K and PLCγ2. Activation of protein kinase A (PKA) has been controversial, but it has been shown that cAMP can modulate both positive and negative CD40-induced responses (28). In recent years many studies have concentrated on the involvement of serine/threonine kinases in CD40 signal transduction: stress-activated protein kinase/c-jun amino-terminal kinase (SAPK/JNK), p38 MAPK, and extracellular signal-regulated mitogen-activated protein kinase (ERK). Contrasting results have been obtained regarding JNK, p38, or ERK activation (29-32). It has been established that members of the TNF family associate intracellularly with different families of signalling molecules, including the ‘death domain’ family and the TNF-R associated factor (TRAF) family, in order to transmit signals (33). One of the downstream events in CD40 signalling and TRAFs recruitment is the activation of MAPK kinases (34). CD40 interacts with TRAF3 (35) and with TRAF2. Induction of NF-kB activation after CD40 cross-linking can in large part be attributed to TRAF2 signalling as both a dominant-negative mutant of TRAF2, lacking an amino-terminal RING finger domain, or a natural inhibitor protein called I-TRAF, can prevent NF-kB activation (36, 37). Finally, two other TRAF proteins, TRAF5 and TRAF6, have also been demonstrated to associate with the CD40 receptor (38, 39). Inactivation of the TRAF5 gene resulted in hampered B cell proliferation and up-regulation of various surface receptors (40). Next to NF-kB, CD40 cross-linking results in the expression/activation of other transcription factors, such as AP-1 and NFAT and members of the Jak-
STAT pathway. CD40 is associated with Jak3, and activation leads to phosphorylation of STAT3 and STAT6, key molecules for cytokines signalling (41, 42).

Moreover, CD40 engagement induces an increase in reactive oxygen species (ROS) levels (43, 44). ROS act as signalling intermediates and one of the important effectors of ROS are the MAPKs JNK, ERK and p38, components of kinase cascades that connect extracellular stimuli to specific transcriptional responses (44-46). Recently, a novel role for apurinic/apyrimidinic endonuclease/redox effector factor (APE/Ref-1) in CD40-mediated B cell activation has been demonstrated by Merluzzi et al (47).

All the signals induced by CD40, are then translated into the activation of specific transcription factors that drive further gene activations.

**TRAF mechanism of action in B cell activation**

Cross-linking of CD40 on B cells results in activation of various members of the TRAF family (TRAF2, 5, 6) (48), which in turn activate MAPK p38 and JNK (34, 49-51) and NF-kB molecules. TRAFs are a major group of intracellular adaptors that bind directly or indirectly to many members of the TNF receptor and the IL-1/Toll-like receptor superfamilies.

At present, six mammalian TRAFs, TRAF1 through TRAF6, have been identified. TRAFs are evolutionarily conserved proteins with homologues found in Drosophila melanogaster (dTRAF1, dTRAF2 and dTRAF3) and Caenorhabditis elegans (ceTRAF) (52). All TRAFs are characterized by a highly conserved motif at the C-terminus, termed the TRAF domain (Fig. 2). The TRAF domain mediates binding to the receptors, formation of homo- or heterodimers, and interaction with a number of intracellular proteins and signalling molecules. The TRAF domain is about 200 amino acids in length and is further divided into a TRAF-N and a TRAF-C domain (53, 54). TRAFs can self-associate as homo- or heterodimers of TRAF1 and TRAF2 or TRAF3 and TRAF5 or TRAF6 and either TRAF2 or TRAF3 (53-55). The trimerization of TRAFs requires an intact TRAF domain (56). Furthermore, the TRAF-C domain has been shown to bind downstream signalling molecules such as TANK and NIK while the TRAF-N domain binds to anti-apoptotic molecules such as c-IAP1 and c-IAP2 (57-60). The amino termini of all mammalian TRAFs, except TRAF1, have a RING finger motif, similar to those found in E3 ubiquitin ligases (61). The RING finger is followed by five to seven zinc fingers (62). The RING finger is crucial for NF-kB activation and the zinc finger domain is essential for JNK and NF-kB activation (36, 63). Interestingly, although both TRAF3 and TRAF5 have an isoleucine zipper domain between the zinc fingers and the TRAF-N domain, only the overexpression of TRAF5 activates NF-kB. So far, the function of this isoleucine zipper domain remains to be elucidated.

Many members of the TNF receptor superfamily bind to TRAFs directly through a cytoplasmic motif termed the TRAF-interacting motif (TIM). The amino acid compositions of the TIMs vary among different TNFRs. However, a major consensus sequence (P/S/A/T)X(Q/E)E can be found in CD40 and OX40, that can bind to TRAF2, TRAF3 and TRAF5 (55, 56, 64, 65). TRAF6 appears to have a unique binding motif P-X-E-X-X-(aromatic/acid residue) that is present in CD40 (55, 64).

To investigate the biological functions of TRAF2, TRAF3 and TRAF6 in CD40 signalling, transgenic mice expressing CD40 mutants that abolish binding to TRAF2 and TRAF3 or TRAF6 were generated (66-68). These studies confirmed the critical roles of these three TRAFs in immunoglobulin isotype switching and affinity maturation, B cell maturation and proliferation, and germinal center formation. However, the physiological roles of these individual TRAFs in these B cell functions remain controversial. Further study of the immune functions of TRAF2, TRAF3 and TRAF6 are hindered by the fact that mice deficient in each of these TRAFs die shortly after birth (69, 70). Nevertheless, TRAF2 knockout mice can be rescued by generating TRF2−/− and TRAF2−/− or TNF-R1−/− and TRAF2−/− double knockout mice. These double knockout mice have a normal primary IgM response, but impaired secondary IgG responses (71). This defect may be attributed to the absence of NF-kB activation upon stimulation of CD40, as observed in the TNF-R1−/−, TRAF2−/− splenocytes. Interestingly, TRAF6 deficient splenocytes also have impaired NF-kB activation upon CD40 stimulation, which suggests that both TRAF2 and TRAF6 are essential for this CD40 signalling pathway (69). The role of TRAF3 in CD40 signalling is enigmatic. In vitro CD40 stimulation of the TRAF3 deficient B lymphocytes results in a normal proliferative response. However, reconstitution of mice with TRAF3−/− fetal liver cells reveals an essential role of TRAF3 in mounting a T-dependent immune response (70).

**APE/Ref-1 and regulation of Pax activity**

APE/Ref-1 is a multifunctional protein involved in apurinic/apyrimidinic endonuclease DNA base repair activity, in proofreading exonuclease activity and in modulating DNA-binding activity of several transcription factors including NF-kB, AP-1, early growth response-1 (Egr-1), p53, Pax5 and Pax8 (Fig. 3) (72-77). The DNA binding activity of these
TABLE I - FUNCTIONAL ACTIVITY INDUCED FOLLOWING CD40 TRIGGERING IN VITRO IS CELL TYPE DEPENDENT.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD40 induced functional activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>Proliferation, CD23 expression, differentiation, isotype switch, Fas expression, selection, IL-6 production</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>Cytokine secretion, NO production, production metalloproteinases, monocyte procoagulant activity and tissue factor expression</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Growth and survival, expression costimulatory molecules, enhanced cytokine production</td>
</tr>
<tr>
<td>CD34+ precursors</td>
<td>Proliferation, development dendritic cells</td>
</tr>
<tr>
<td>T cells</td>
<td>Proliferation, CD25 expression, cytokine production</td>
</tr>
<tr>
<td>Endothelial cell lines (see Huvec)</td>
<td>Up-regulation CD54, CD62E and CD106, increase tissue factor/thrombomodulin expression and procoagulant activity, T cells costimulation, increased production LIF, IL6 and GM-CSF</td>
</tr>
<tr>
<td>Vascular endothelial cells</td>
<td>Increased IL1, IL 6, IL 8 production</td>
</tr>
<tr>
<td>Thymic epithelial cells</td>
<td>GM-CSF production</td>
</tr>
<tr>
<td>Kidney epithelial cells</td>
<td>Cytokine/chemokine secretion: IL 6, LIF, GM-CSF, IL 8, MCP-1, RANTES</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>Enhanced expression CD54 and Bcl-x, IL 8 secretion</td>
</tr>
<tr>
<td>Carcinomas and transformed epithelial cells</td>
<td>Growth inhibition/apoptosis</td>
</tr>
<tr>
<td>Synoviocytes</td>
<td>Proliferation, cytokine production (GM-CSF, MIP-1α)</td>
</tr>
<tr>
<td>Lung fibroblast</td>
<td>Increased IL 6, IL 8 and NF-kB increased Cox-2, PGE2</td>
</tr>
<tr>
<td>Dermal and Thyroid fibroblast</td>
<td>Increased IL 6, IL 8, NF-kB</td>
</tr>
<tr>
<td>Follicular dendritic cells</td>
<td>Growth, CD54 expression</td>
</tr>
<tr>
<td>Vascular smooth muscle cells</td>
<td>Increased MMP-1, MMP-3, MMP-9, MMP-2, activation ICE (IL-1β converting enzyme), induction stromelysin-3 (MMP-11)</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Induction of Fas-L expression</td>
</tr>
</tbody>
</table>

proteins is sensitive to their reduction-oxidation (redox) status. APE/Ref-1 represents a novel component of signal transduction processes that regulate eukaryotic gene expression. Specifically, apurinic/apyrimidinic endonucleases enzymes exhibit strong hydrolytic activity and 3'-diesterase activity on apurinic/apyrimidinic nucleotides with APE/Ref-1 having the highest 5'-endonuclease rate, but lowest 3'-diesterase activity. The gene encoding the APE/Ref-1 protein maps to chromosome 14 bands q11.2-12 in the human genome (78). The complete gene is 2.6 kb and consists of four introns and five exons, the first of which is non-coding. The APE/Ref-1 protein is modest in size, it is 318 amino acids in length and ~37 kDa. It contains two distinct domains. The N-terminal domain contains the nuclear localization sequence (residues 1-36) (79), and is essential for redox activity, while the endonuclease activity resides in the C-terminal region (80). Since APE/Ref-1 is an important protein for cellular survivability, it was expected to be ubiquitously expressed in cells. Indeed, APE/Ref-1 expression is ubiquitous, however, it exhibits a complex and heterogeneous staining pattern that differs among tissue types and within the cells shows nuclear and/or cytoplasmic localization (81). APE/Ref-1 is regulated at both the transcriptional and post-translational level. In terms of transcriptional regulation, the effects of ROS on APE/Ref-1 induction have been the most intensely studied. In both in vivo and in vitro studies, oxidative agents induce APE/Ref-1 (74, 82). Induction is characterized by a transient increase in APE/Ref-1 protein and mRNA. The process of APE/Ref-1
induction by ROS may involve two steps. In the first step, APE/Ref-1 translocates from the cytoplasm to the nucleus (74, 82). In B lymphocytes, the translocation is fairly rapid; it occurs within an hour (74), whereas in HeLa cells the process is on the order of hours (82). The second step involves de novo protein synthesis via transcriptional activation of the promoter (74, 82). Given that ROS are generated endogenously as by-products of cellular respiration and also during such pathological states as reperfusion injury and inflammation, the induction of APE/Ref-1 seems an important response to maintain cellular homeostasis. APE/Ref-1 functions as a 3'-diesterase in the repair of ROS-mediated damage by generating 3'-hydroxyl primers for β-pol. It has been postulated that APE/Ref-1 may be the rate-limiting step in repairing ROS-induced damage (82) and, thus, ROS-mediated induction of APE/Ref-1 may be a compensatory mechanism. Alternatively, APE/Ref-1 may regulate gene expression as a component of a cell response to oxidative agents. APE/Ref-1 stimulates the transcriptional activity of numerous transcription factors that have physiological functions as diverse as cell cycle control, apoptosis, angiogenesis, cellular growth, cellular differentiation, neuronal excitation, haematopoiesis and development. In 1992, Xanthoudakis and Curran (75) identified APE/Ref-1 as an important redox activator of the DNA binding of transcription factors Fos and Jun (AP-1 subunits) (75). It was also discovered, through mutational analysis, that the conserved cysteine residue located in the DNA binding domain of Fos and Jun was essential for the APE/Ref-1-

<table>
<thead>
<tr>
<th>TF</th>
<th>Name</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
<td>Cell growth and differentiation, stress response</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kB</td>
<td>Cell growth and differentiation, stress response</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclin AMP response binding protein</td>
<td>Cell growth and differentiation, stress response</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcriptional factor</td>
<td>Cell growth and differentiation, stress response</td>
</tr>
<tr>
<td>myb</td>
<td>Myb</td>
<td>Hematopoiesis</td>
</tr>
<tr>
<td>Pax</td>
<td>Paired box</td>
<td>Hematopoiesis</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor 1</td>
<td>Response to hypoxia</td>
</tr>
<tr>
<td>HLF</td>
<td>HIF-like factor</td>
<td>Response to hypoxia</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Early growth response-1</td>
<td>Myeloid cell differentiation</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Nuclear factor-Y</td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>p53</td>
<td>p53</td>
<td>Response to stress, cell cycle regulation and apoptosis</td>
</tr>
<tr>
<td>PEBP2</td>
<td>Polyoma virus enhancer-binding protein 2</td>
<td>Hematopoiesis</td>
</tr>
</tbody>
</table>
mediated activation of AP-1 (75). While the exact nature of the redox change is not known, it is known that oxidation of the cysteine residue abolishes DNA binding, whereas reduction to a sulfhydryl state promotes DNA binding. APE/Ref-1 has been shown to activate DNA binding via the reduction of a cysteine residue of numerous transcription factors, such as activating transcription factor (ATF), CREB, the oncogene Myb, nuclear factor-Y (NF-Y) and Egr-1 (76, 77, 83) (Table II).

The DNA binding activity of the Pax gene family, is under redox control and is regulated by APE/Ref-1 (47, 74). Thus, APE/Ref-1 could act as a signalling intermediate in B cell activation and in the regulation of immune responses, linking extracellular signals with the regulation of key target genes for B cells activation process. Indeed, co-transfection of APE/Ref-1 with Pax5 results in an increase in the Pax5-induced transcriptional activation of the B cell specific activator protein promoter, the CD19 promoter (74). APE/Ref-1 controls the redox state of the Pax5 PAI sub domain. The DNA binding activity of the RED sub domain is not subject to redox control. Pax5 binds DNA when the PAI is oxidized and, conversely, it does not when PAI is in a reduced state. This level of control presumably allows Pax5 to activate various target genes, depending on its redox state. Consequently, APE/Ref-1, by virtue of its redox control of the PAI domain, also indirectly controls the DNA binding activity of the RED domain (84).

Merluzzi et al., have recently shown that CD40 signalling depends on the redox APE/Ref-1 factor: upon CD40 antibody-mediated cross-linking, APE/Ref-1 translocates from the cytoplasm to the nucleus of activated B cells, where it modulates the DNA binding activity of both Pax5a and EBF (47). It is known that different TRAFs exhibit specific biological functions, but the role of individual TRAFs in the activation of different CD40-dependent signalling pathways has not yet been defined. Therefore, in the present study, we investigate the role of TRAF molecules and TRAF-activated MAPK triggered by CD40 in the APE/Ref-1 nuclear translocation.

**OX40L structure, function and signalling pathway**

OX40L on activated B cells, represents a co-stimulatory signal, which promotes continued proliferation and clonal expansion of CD4+ T cells during the later phase of immune response and maintains T-cell long-term survival (85, 86). OX40/OX40L pair of complementary molecules is a two-
way signalling system, with OX40 on activated T cells providing a differentiation signal to B cells (87, 88), and OX40L on B cells having the potential to costimulate T cells (89).

There is a close relationship between CD40 stimulation and OX40L expression: CD40 stimulated B and DCs express OX40L within 2 days of stimulation and are able to sustain this expression beyond day 7 (90). Since CD40 signalling is known to be upstream in the pathway for NF-kB activation (36), Murata and colleagues findings depicting the requirement of CD40 stimulation in the induction of OX40L expression, are consistent with those of Ohtani and colleagues, showing the downstream upregulation of the promoter activity of OX40L by NF-kB activation (91).

OX40L, the ligand for OX40, belongs to TNF superfamily as well as CD40L, and was originally identified as a type II transmembrane glycoprotein of 34 kDa (gp34) expressed on human T-cell leukemia virus type 1 (HTLV-1)-infected T-cell lines. It is mainly expressed on activated APCs, such as activated B cells, mature DCs, activated macrophages and endothelial cells, but it has also been found on activated OX40<sup>−/−</sup> T cells (87, 88, 92-94).

OX40L is supposed to be an homotrimeric protein composed of three jelly-roll subunits and is one of the most divergent members of the TNF superfamily.

The OX40-OX40L interaction has been shown to induce bidirectional signals and thus is implicated in various immunological responses. (i) OX40L on DC provide a costimulatory signal to OX40<sup>+</sup> helper T cells, resulting in increased proliferation, cytokine production, and preferential differentiation of naïve CD4<sup>+</sup> T cells to Thelper2 phenotype. Simultaneously, ligation of OX40L on human DC enhanced their maturation and production of cytokines (92). (ii) OX40L expressed on activated B cells can receive a signal from OX40 on helper T cells, which resulted in B-cell proliferation and immunoglobulin secretion (87, 95); and (iii) the OX40/OX40L system directly mediated the adhesion of OX40<sup>+</sup> T cells to OX40L human umbilical vein endothelial cells (HUVEC) (93). Stuber et al., have shown that cross-linking of OX40L with OX40-Ig induces the proliferation and IgG secretion by CD40L-stimulated B cells (87). Moreover, they showed that Pax5 levels and its DNA binding capacity are down-regulated by 60%-80% in response to OX40L cross-linking (87). Actually, the transcription factor Pax5 has two binding sites in the immunoglobulin *heavy chain* gene 3′ α enhancer, occupation of which represses this enhancer (96). Since Pax5 is not expressed in plasma cells, the enhancer is active in these cells, which is believed to be important for the high level immunoglobulin secretion of plasma cells (96). Stuber and colleagues suggest that at least two T cell-B cell surface interactions must occur before the B cell can produce large amounts of IgG antibodies in response to a T cell-dependent antigen, one involving CD40L-CD40 leads Ig isotype switching and is crucial for all subsequent B cell differentiation events; another involving OX40-OX40L allows B cells to produce large amounts of Ig (88). OX40L

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**Fig. 2 - Domain organization of the mammalian TRAFs.** R, RING domain; Z, zinc-finger domain; TRAF-N, coiled-coil domain; TRAF-C, TRAF-C domain. The TRAF domain comprises the coiled-coil domain and the TRAF-C domain.
knockout (OX40L<sup>−/−</sup>) mice are viable, and the genotypes of progeny resulting from heterozygous intercrosses followed normal Mendelian ratios. Flow cytometric analyses reveal comparable numbers and maturation of thymocytes in wild-type and OX40L<sup>−/−</sup> mice as well as comparable numbers of B cells, T cells, and T cell subsets. Expression of the activation markers CD25 and CD69 is also similar. These results suggest that OX40L is not crucial in the early development of T and B cells (97). However in OX40L<sup>−/−</sup> mice, both T cells and B cells with memory phenotype are significantly reduced after repeated alloimmunizations. Moreover, OX40L on B cells is not required for memory CD4<sup>+</sup>/T-cell function, but is critical for memory B-cell development and/or survival after repeated alloimmunizations (98). OX40L is induced during immune activation, and the inducible mechanism of the OX40L gene is strictly controlled. OX40 is also inducible, unlike other members of the TNF receptor family (FAS, TNF receptor, and CD40). Thus, production of OX40L may be important in either maintaining or terminating immune reactions. With the exception of Ig isotype switching, which is made possible by stimulation through CD40 and guided by cytokines (99), the specific signals that drive a B cell towards terminal differentiation on the one hand or memory development on the other, have yet to be identified.

**Regulators involved in the terminal differentiation of B cells**

Several transcription factors have been implicated in plasmacytic differentiation. IRF-4, a hematopoietic transcription factor, is expressed in plasma cells and a small number of germinal center cells that lack Bcl-6 (a transcriptional repressor required for GC B cells and downregulated in plasma cells), suggesting that it is induced upon plasmacytic differentiation (100). However, IRF-4<sup>−/−</sup> B cells fail to proliferate normally in
response to LPS in vitro (101). Both Bcl-6 and the B lymphocyte induced maturation protein (Blimp-1) can block activation by IRF-4 making the role of IRF-4 unclear in terminating a GC reaction and inducing a plasma cell fate (102, 103). Blimp-1 is a transcriptional repressor that has been called a “master regulator” of plasma cell differentiation. It is a 98-kDa protein containing five zinc finger motifs, which are responsible for sequence specific DNA binding (104). Blimp-1 can associate with hGroucho, histone deacetylases, and the G9a histone methyltransferase as part of its repressive function (105-107). Blimp-1 is induced in sorted marginal zone B cells stimulated with LPS in vitro within 24 h (108). In vivo, Blimp-1 is seen in early plasma cells in the red pulp of the spleen, in a small number of GC cells that have some plasma cell characteristics, in bone marrow plasma cells, and in IgG+ plasma cells in a memory response (109). However, Blimp-1 was not seen in CD20+CD38- human tonsillar memory cells (109). Blimp-1 is sufficient to induce plasmacyct differentiation of mature B cells in vitro as well as in B cell lines (110-112). It acts by inducing a cascade of molecular events that both enforces plasmacyct differentiation and inhibits B cell identity (111). To achieve this, Blimp-1 regulates three large gene expression programs by direct repression of a transcription factors (111). Direct repression of Pax5 is particularly important because of the role for Pax5 in regulating so many genes involved in Ig transcription, BCR signalling and B cell identity. Blimp-1 blocks a proliferative program, in part through direct repression of c-myc (113), consistent with cessation of cycle in plasma cells. Blimp-1 also causes upregulation of genes involved in Ig secretion including Ig heavy and light chain genes and XBP-1, in part through repression of Pax5 (114). Finally, Blimp-1 represses a large number of genes required in mature B cells including genes involved in GC functions (Bcl-6, AID, DNA-PKcs, Ku70, Ku80, Ku86), intracellular signalling (syk, btk, BLNK, lyn, fyn), receipt of extracellular information (Igκ, MHCII, CD19, CD21, CD69, CD86), and follicular homing (CXCR5) (111). However, despite this wealth of knowledge regarding the role for Blimp-1 in plasma cell formation, it is still not clear if Blimp-1 is required for the terminal differentiation of B cells into Ig secreting cells.

Another transcription factor, XBP-1, was the first transcription factor found to be required for plasma cell formation (115). Additionally, XBP-1 may be required for plasma cell maintenance since the proteasome inhibitor PS-341, which can induce apoptosis in multiple myeloma cells, acts in part by preventing the activation-induced mRNA processing of XBP-1 message (116). XBP-1 is thought to act after Blimp-1, since Blimp-1 is expressed normally in B cells lacking XBP-1 (115) and since Blimp-1 can upregulate XBP-1 by direct repression of Pax5 (114, 117). XBP-1 is an important factor in the mammalian unfolded protein response (UPR), a series of molecular events that allows cells to cope with large amounts of protein in the endoplasmic reticulum (ER) (118). Indeed, XBP-1 is required for Ig secretion (119), but the initial ER expansion in plasmacytic differentiation occurs before XBP-1 is at high levels (120) and in the UPR in mouse embryonic fibroblasts, XBP-1 is only required for the upregulation of some UPR genes (121). Thus, it is not clear to what extent the preparation for Ig secretion required XBP-1. Additionally, Blimp-1 would be likely to play a role in this process due to its induction of XBP-1 and other factors, but the degree of involvement is yet unknown (122).

It is believed that when B cells commit to the plasma cell differentiation pathway, the gene expression program that defined their naïve B-cell identity is repressed and a program that drives them on a one-way street to terminal differentiation is turned on. Unlike in plasma cells, in memory B cells Pax5 is crucial for maintaining naive and memory B-cell identity (123), as it activates genes required for maintenance of B-cell identity (e.g. CD19 and BLNK) (124), while simultaneously repressing genes required for plasma cell differentiation and antibody secretion (e.g. XBP-1) (117).

However, despite the discovery of new molecular actors in the signal transduction pathway theatre of B cell activation we are far from having a coordinated view of the molecular events that lead to the generation of effectors B cells.

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Outside inside signalling in CD40-mediated B cell activation

Taurine-Diabetes Interaction: from involvement to protection

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ABSTRACT: Taurine is a sulfur amino acid (2-amino ethane sulfonic acid) and has been claimed for a number of beneficial actions ranging from anti-epilepsy to anti-hypertension. Taurine in diabetes has an age old story; taurine is involved in the development and protection of insulin apparatus. Taurine and insulin both have mutual stimulating actions with hypoglycemic properties. On the clinical front, taurine supplementation has an acceptable beneficial effect in platelet aggregation and, to name few more, in neuropathy, cardiomyopathy, and nephropathy to retinopathy. Recent studies have provided a role for taurine in fetal development and in blocking the transfer of diabetes from diabetic mother to offspring. A number of mechanisms for the actions of taurine have been advocated, from osmoregulation to anti-oxidation. Though sulfonyleurea and recently introduced thiazolidinediones are effective, however they are not free from complications, thus there is a need to design new therapeutics. As taurine is also a sulfonyle derivative, it will be of great interest to develop taurine analogues as an alternative therapy. Considering the great involvement of taurine in diabetes, this review may provide a holistic view of taurine in diabetes and in its prevention in this century.

KEY WORDS: Taurine and Diabetes

INTRODUCTION

Taurine is the most abundant free amino acid in mammals, and it is in almost all important tissues such as brain, liver, kidney, eye and heart. A normal human of about 70 kg contains about 0.1% taurine. This high amount of taurine provides logic for its greater involvement in almost all life processes from the functioning of eyes to ears and lungs to liver. For this great involvement taurine has been given many names, from a preventive medicine (1) to a vitamin-like molecule (2). In diabetes, nutrients play a vital role, as much of the recent surge in diabetes can be blamed on changing eating habits, hence the cure of diabetes lies also in nutrients, and happily, a steady supply of such nutrients has formed to alleviate diabetes: taurine has been placed in this category for some time (3-5). The study of taurine actions reveals that taurine is found to stimulate glycolysis and glycogenesis (6) and is reported to have insulin-like action (7, 8). A number of beneficial effects of taurine are recorded every year, ranging from reducing liver injuries (9) to islet cryo-preservation (10), and many findings on its involvement in bile acids and related activities (11), and many more on neurological and urological actions to retinal and fetus development (12). A large number of clinical trials make it suitable for use as a therapeutic tool (13, 14).

In type two diabetes, the treatment of hyperglycemia, in addition to insulin, also needs some other agents. Currently such agents are sulfonyleureas. Sulfonyleureas often show toxic effects and often cause hypoglycemia. A new group of agents, thiazolidinediones, was recently introduced, but also causes several complications (15). The fast changing environment and living style further complicate the complex nature of diabetes. Thus, to cope with this, new therapeutics are badly needed (16). With taurine being a sulfonyl compound, it has the potential to provide an alternative to sulfonyleureas, if some of the limitations of the taurine such
as limited permeability, poor turnover rate and poor absorption, were minimized through development of lipophilic analogues; taurine analogues may provide more effective therapeutics.

Taurine: biosynthesis and regulation

Almost all mammals have no or very little synthetic ability; liver has a little synthetic ability and the synthesized taurine is distributed to other organs (17). There are several pathways for taurine biosynthesis starting cysteine. In brief, this can be represented in Fig. 1. Taurine regulation is a combination of biosynthesis, intake and excretion, whereas biosynthesis is almost nil, thus regulation has to provide an equilibrium between intake and excretion to a process of accumulation, release, uptake, transport, and metabolism. Accumulation of taurine is mostly through dietary sources and via an active transport system: release of taurine is an independent phenomenon but the rate of release, is cation dependent, whereas uptake is sodium dependent, and temperature sensitive. Recently, the role of taurine transporters has come to prominence (18). In general, metabolism of taurine is a slow process but during reduced dietary supply, the rate of taurine excretion is extremely slow. The availability of taurine can be modified by altering dietary supply (19, 20) and by use of an antagonist. In the absence of any effective antagonist, taurine transport inhibitor GES or β-alanine are mostly used, which effete-ly reduce the taurine content up to 80% in a few weeks (21, 22).

Taurine contents of major organs in diabetes

Normally in a healthy person, taurine concentration is high in the heart, brain and muscles. The heart contains about 60% of free amino acids, as taurine. A study performed by Learn et al (24) on the taurine content of human blood reported platelet has 5 nmols/1×107 cells, plasma 30 nmols/1×107 cells and serum 129 nmols/1×107 cells. Taurine contents are altered in diseased states, and a study (24) on the taurine content of normal and diseased platelets, found 261 μmol/1012 platelets in normal condition, 323 μmol/1012 platelets in congestive heart disease, 231 μmol/1012 platelets in hypertension, and 213 μmol/1012 platelets in myocardial infarction. Taurine contents under diabetic conditions have provided mixed results. An early study (25) reported higher taurine content in amniotic fluid in diabetic pregnancies. An increased urinary taurine excretion in diabetes was also observed (26) as 17.2 in non-ketotic and 18.4 in ketotic in comparison to a reference group (13.2 Mm mole/24/h BSA). Franconi et al (27) also found lower taurine in type I diabetes, however, newborn infants of diabetic mothers has recorded elevated plasma taurine concentration (28). A recent detailed study by Deluca et al (29, 30) reported lower taurine level in plasma of 28 type II diabetic patients. In Type I diabetes the same trend was also observed, with platelet taurine uptake being significantly reduced. The reduced uptake under diabetes is believed to be due to a selectively impaired transport system.

Pharmacological actions of taurine in diabetes: an overview

The high concentration of taurine tends to maintain the cellular function normal, even under diseased conditions. Though not many studies have been reported on diabetic conditions to date, the pharmacological roles of taurine in major organs like heart, brain, kidney, and retina are well documented. It is believed that taurine in diabetes acts via osmo-protection and helps to maintain cellular identity, in addition to this it also act via host defense, scavenging reactive oxygen species. Taurine also possess radioprotection property (31). Taurine prolongs the life of 56Co irradiated mice and also promotes leukocyte recovery in radiation exposed mice (32). Taurine derivative, glutamyl-taurine, has prevented the development of hyperglycemia, caused by X-ray irradiation (33). Taurine supplementation has also increased the taurine contents of irradiated mice (34).

Scavenging, and host defense

A large number of nutrients act as antioxidants, such as vitamin C, E, flavonoids and taurine. In diabetic conditions, the level of antioxidant falls below the normal level, creating the adverse situation with an increase in free radicals. Taurine takes part in the inhibition of such free radicals, which are also known as reactive oxygen species (ROS) and taurine may act as a trap for such radicals: such reaction may follow the pathway as depicted in Fig. 2. In this transformation reaction, taurine chloramines are formed which are less reactive and much less toxic than HOCl. In a similar manner, taurine reacts with other chlorinated oxidants and reactive carbonyl compounds, hence arresting the lipid per-oxidation. In diabetes, taurine reduces the accumulation of lipid per-oxidation by reducing the amount of malondialdehyde and another reactive carbonyl compound, 4- hydroxy alkenal. Taurine also reduced the glomerulopathy in experimentally induced diabetic rats through drinking water supplementation (35). Changing environmental factors produces a number of toxic pollutants, making cell risk prone and subject to oxidative stress. This may eventually lead to a harmful effect on the body.
through further oxidation, directly or indirectly provoking inflammation. To minimize these effects, endogenous antioxidants or supplementation of such antioxidants may be useful. With taurine being an endogenous antioxidant, it functions well in cytosol of human leukocytes and acts as a trap in scavenging reaction in a similar manner to reactive oxygen species, and produces less reactive and less toxic substance. Thus, taurine acts as an endogenous antioxidant and constitutes part of host defense (36).

Osmo-protection is another way by which taurine participates in cellular homeostasis, which is one of the oldest actions of taurine. Due to the ionic imbalance across the membrane, membrane excitation results in altered cell volume, which may finally lead to the rupture of cell membrane and disintegration of cellular identity. To avoid such a situation, cellular contents, especially inorganic ions and osmolytes, must be modified to minimize the effect of the osmotic stress (37). Osmo-regulation function of taurine has been well studied in brattleboro rats with hereditary hypothalamic diabetes insipidus (DI) (38).

Apart from the above-mentioned two major cellular protections, taurine has cardio tonic action: cardiovascular diseases are the most observed phenomenon in diabetes mellitus (39, 40) with altered energy metabolism in diabetic heart. Taurine has been shown to potentiate the glucose utilization in heart (41). In experiments on rat heart (42), it was found that the ability of taurine to modulate myocardial contraction and calcium transport may be through the inhibition of phospholipid - N methylation. An increase of calcium content in diabetic myocytes by taurine has also been monitored (43).

Besides all the above, taurine does also have some other actions, like attenuation of hypertension and improvement insulin sensitivity in fructose fed rats (44), reduction of hypercholesterolemia (45) and improvement of hepatic circulation (9, 46).

**Taurine and insulin in symbiosis**

Both taurine and insulin exert modulatory action on release and potentiation of actions of each other. Taurine specifically binds to insulin receptor in a reversible manner (47). The insulin stimulating action of taurine includes enhanced formation of taurine containing bile salts (48). It also stimulates taurine uptake in rat retina (49). Topical insulin also increased the taurine level during reperfusion in streptozotocin induced hyperglycemic ischemia in rat cerebral cortex (50). In humans, insulin infusion resulted in increased taurine level and improved myocardial metabolism (51). Taurine exerts insulin-like, insulinogenic actions, liberating insulin from the pancreas (7, 8, 31). It is suggested that the insulin-like action of taurine, is via cAMP. A role of taurine in the regulation of insulin secretion from islets was also stressed (52). Taurine also improves insulin sensitivity in Otsuka rats, a model of type II diabetes (53). In type II diabetes, taurine involvement in enhancement of bile formation may result in improved lipid metabolism and insulin resistance.

Insulin deficiency is a condition which is observed in hyperglycemia, and is a sign of diabetic state. This phenomenon is well documented in type II diabetes and it can be corrected by insulin supply, however in type II diabetes, insulin treatment only partially normalizes the enhanced glucose level. As hyperglycemia is caused by insulin resistance, insulin resistance can be minimized in part through its increased secretion from the pancreas. Insulin secretion mainly depends on glucose challenge, amino acid content (of which taurine is very high) and amount of bile salts (54). A higher taurine content may provide a higher amount of bile salts also. A high concentration of taurine may provide an osmo-regulatory role of taurine in α cells and a modulatory role on release of insulin secretion (55) from pancreatic β cells, hence helping to create a hypoglycemic state. The hypoglycemic effect of taurine is the one of its oldest recorded effects in diabetes (56). Hyperglycemia causes protein glycation subsequently producing advanced glycation end products (AGE) (57, 58). Chemically, AGE are formed by the reaction between a free amino group with a carbonyl group of sugar like glucose and fructose, producing 1-amino 1-deoxyketose; Amadori product, a carbonyl compound. This mono carbonyl intermediate on further oxidation produces very reactive dicarbonyl compound, such carbonyl compounds are responsible for glycosylated proteins, common in diabetes. It is believed that taurine acts in scavenging these carbonyl compounds (59), but in taurine-depleted conditions sorbitol accumulation will be higher, leading to increases in AGES and carbonyl group containing compounds hence enhancing the diabetic complication.

**Taurine under experimental diabetes studies**

Perfection and precession are the tools to providing maximum results; to follow this, mice strains C57B2/6 or C57B2/6J or C57/6 are abnormal in taurine homeostasis with urinary taurine extraction of 3 to 10 fold higher due to very poor renal resorption are available. One of these strains, C57B2/6, has dysfunction of taurine homeostasis and it is a role model for diabetic-taurine interaction.
Taurine supplementation and depletion studies
Studies of the effects of taurine under various conditions are necessary to conclude the role of taurine and its significance. To date, studies have been mostly carried out on rats and mice with chemically induced diabetes. In such conditions, taurine was given orally or through drinking water ranging from 0.5 to 5% or through dietary supplement (60, 61). Similarly, depletion was done using β alanine in drinking water (62). Streptozotocin induced diabetic or high cholesterol fed rats attenuated vasodialation, but taurine supplementation reversed it. Taurine supplementation protects type I diabetic mice from lipid per-oxidation (63). Taurine supplementation also helps to restore integrity of pancreatic β cells and maintain normal secretory granules; however, non-taurine supplemented diabetic rats were found to have severely damage secretory granules (64). The insulin levels in pancreatic β cells of a diabetic group with no taurine supplementation were markedly reduced but 1-2% supplement of taurine elevated the insulin levels (62).

Platelet aggregation
Platelet accumulates quite good amounts of taurine. Platelet aggregation in diabetics has been clinically studied more than in experimental animals. In one of the experimental studies, in brattleboro diabetes insipidus rats (65), the hyper-aggregation of platelets has been recorded, it is believed to occur via polyol pathway involving aldose reductase inhibition (37). Anti-platelet aggregating effect of taurine has also been observed in cats (66).
Diabetic cardiomyopathy
Cardiovascular diseases are the major constituents of diabetic complications. Such diabetic complications may result in myocardial infarction, stroke and gangrene but the most troublesome is diabetic cardiomyopathy (67). Taurine level in diabetic heart is generally high (68, 69). In experimental diabetes, taurine level was recorded 30% higher in heart than control but such observation was mostly recorded in type-1; however, in type-2 it is mostly unchanged. The increased taurine may be responsible for maintaining plasma osmolity, and thus tends to maintain homoeostasis. Diabetic cardiomyopathy has been related to up regulation of the renin angiotensin system (70) and taurine has been reported to down regulate angiotensin-induced hypertrophy in myocytes (11).

Diabetic nephropathy and retinopathy
In diabetes, the kidney is the worst affected organ and it is the first affected part, resulting in renal dysfunction with increased amount of albumin in urine, creating osmotic stress. It is believed that taurine along with other osmolytes has a shielding effect on osmotic stress and thus, helping in normalizing renal dysfunction. In a study on STZ induced diabetic rats, decreased renal taurine contents have been recorded (72, 73). The immuno-histological studies have located taurine in rat medullary tubules and taurine pool is maintained by modulating proximal tubules re-absorption.
of taurine as per dietary supply. In STZ induced diabetes, taurine has been observed to spread in larger area covering most medullary tubules rather than limited to proximal tubules and glomeruli region as in normal rats. It seems that this extended area of taurine distribution may help in preserving renal functioning in diabetes (74). Taurine supply in STZ induced diabetes rats may help in ameliorating diabetic nephropathy through decreased lipid peroxidation and reducing the AGE in kidney. Taurine’s beneficial effects were further observed, when high glucose inhibits renal proximal tubule cell proliferation and induces oxidative effects, but these effects of glucose were blocked by taurine (75). Eyes are the first affected organ in diabetes.

Diabetic lens accumulate sorbitol, producing osmotic stress, with a decreased amount of taurine and myo-inositol. Taurine’s beneficial effect has been observed in diabetic cataracts, where sorbitol accumulation in lens fibrils causes swelling due to...

Fig. 4 - Taurine-Diabetes interactions and beyond.
exogenes of osmolytes and movement of water to occupy the vacant space; this excess water causes disruption of fibrils, resulting in a cataract (76). Taurine’s preventive effect in diabetic cataract formation was further examined (77, 78) in isolated rat lenses. An anti-oxidation mechanism of taurine in preventing cataract lens has been advocated (79, 80).

Arthroscleroses, endothelium dysfunction and protection of liver injury

Besides affecting the vital organs, diabetes also distributes the other cellular activities, for example when cholesterol extraction is reduced, the lipid forming fatty acid adheres to the walls of the arteries creating atherosclerosis. Arthrosclerosis is associated with low production of bile acids (81). Arthroscleroses can develop when high-density lipoprotein (HDL) cholesterol in plasma decreases, but dietary taurine has an increasing effect on its level (82). In diabetes, vascular lesions are more visible due to morphological abnormality in endothelium; the severity of hyperglycemia is related to such changes in endothelium. A high-level glucose is generally toxic to endothelial cells resulting in cellular dysfunction. However, a chronic administration of taurine has a tendency to normalize endothelium-dependant cellular activities (83). Taurine also has a beneficial effect in reducing liver injury in rats (9, 46).

Infant and diabetic mother-disease of generation

The role of taurine in infants and diabetics is a recent addition. It has been observed that the offspring of a diabetic mother may show diabetogenic effect. They may develop abnormal pancreatic islets producing insufficient insulin resulting in type1 diabetes, or may have impaired glucose metabolism as in type-2 (84). Such conditions may also complicate diabetes in later stages with severe heart and related diseases. In a study with rats (12, 85), it was found that taurine level is lower in diabetic mothers, their fetus and offspring, and a generation later. The low levels of taurine and other neuro-active amino acid like GABA, may be involved in the hypothalamohypophyseal regulation of insulin secretion, producing insufficient insulin, leading to the development of impaired glucose metabolism and gestational diabetes, carried out generation to generation. Reusens and Remacle (86) have carried a good review of this subject.

Diabetes and neural dysfunctions

The effects of diabetes on the brain and related organs and their functions are not in immediately noticeable, but a variety of neural problems develop in later stages of diabetes leading to neural dysfunction. Polyol pathway is involved in these dysfunctions in which important osmolytes, like myo-inositol and taurine, are depleted (87). The depleting osmolytes cause defective volume regulation, leading to disturbances in neural transmission and resulting in neural dysfunction. As taurine is regarded as a non-perturbing organic osmolyte and protects brain cells from dehydration avoiding the hypernatremia, in a similar manner, its also limits the effect of hyperglycemia arising from insulin deficiency resulting from hyperosmobilty (88). In such situations, these osmolytes also tend to prevent brain cell shrinkage. The role of osmoregulation was further stressed by Stevens et al (87) through their experiments in glucose-induced sorbitol accumulation in diabetes mellitus which resulted in taurine depletion, followed by impaired nerve regeneration with neuronal hyper excitability and pain. A detailed study of experimental diabetic neuropathy has been also recorded (8, 88, 89).

Taurine in clinical implications

Diabetes may combine more than one disease, but the most affected are of vascular and neural origin, including neuropathy, nephropathy, retinopathy, cardiomyopathy, platelet aggregation and atherosclerosis, creating systematic conditions, leading to vascular or endothelial dysfunctions (90). Diet management and uninterrupted insulin therapy prolongs the appearance of complications, but in almost all cases it does happen in later stages. Several experimental data had provided beneficial effect of the utility of taurine in various diseases combined with diabetes as well as alleviating the hyperglycemia. Such beneficial actions in experimental models provided strong support for clinical utilization, where also beneficial effects of taurine were recorded, a few of which are mentioned below:

Platelet aggregation

Platelet aggregation is the first to develop in diabetes which, with time, aggravates further to become hyper-aggregation. Taurine has a lowering effect on platelet aggregation. In diabetic patients, taurine levels of platelets are lower (148/183 nmol/mg protein, diabetic/control). Platelet taurine uptake in diabetic patient is also very slow; however, taurine release is fast (29, 30). With an oral taurine supplementation of 1.5g/d for 90 days in an IDDM patient, the plasma and platelet taurine concentration increased significantly to a level almost equal to healthy subjects. An effective dose of arachidonic acid required for platelets to
aggregate was much lower in diabetics than in controls (27); it is further stressed that a decreased level of platelet aggregation is a consequence of increased level of taurine (66).

**Cardiomyopathy**

The experimental success with taurine in cardiomyopathy in diabetics, prompted a clinical study. In such study by combining western and traditional Chinese medicine in acute viral myocarditis (14), 48 patients were divided into two groups, one on combined Chinese and western medicine including *Astragalus membranaceus*, taurine, coenzyme Q10 and antiarrhythmics, while others received Gik (Glucose - insulin - potassium), coenzyme Q10 and antiarrhythmics. The combined therapy has an edge over regular therapy in improving the clinical manifestation, however, more such studies are required to prove the efficiency of taurine.

**Diabetic nephropathy**

Renal dysfunctions are very common in chronic diabetic patients. Ameliorating effect by taurine in such chronic diabetic rats are well known. Patients with uremia and on chronic dialysis have been found to have low levels of taurine (91-92). A taurine supplementation study in diabetic patients with microalbuminuria has provided very significant improvement (93). Alvestorend et al (94) also recorded an abnormal level of amino acid in ureamia. An over extraction of taurine in renal glucosuria was further observed, which may be due to genetic defects (95). Low plasma taurine was further recorded in chronic renal failure in patients with diabetes mellitus (96).

**Infant and diabetic mother**

Taurine involvement in infants and diabetic mothers is one of the earliest known taurine-diabetes interactions in a clinical study. In one study (25), it is observed that the concentration of taurine is below normal in diabetic pregnancies. A study on diabetic children showed increased excretion of taurine in ketotic diabetes (26), but a decreased urinary excretion of taurine was recorded in diabetic ketoacidosis in children (97). This fluctuation may be due to an altered metabolic state and renal dysfunction, probably in re-absorption of taurine. Recent studies have shown that alteration in the placental transport of amino acid may contribute to an impaired fetal growth in pregnancies complicated by diabetes (98). An increased uptake of neutral amino acids across syncytiotrophoblast microvillous plasma membrane may be used in placental metabolism and may be delivered to the fetus. Thus, the role of taurine in pregnancy and after is increasing, hence during pregnancy an adequate pool of taurine in maternal tissue is necessary which can be released during the prenatal period to the fetus via placenta and to the new born through the mother's milk. A low maternal taurine will lead to a level of low fetal taurine, and such taurine deficiency may affect normal fetus growth, nervous development and endocrine pancreas (85). Such offspring at adulthood may have impaired neurological function, glucose tolerance, and vascular dysfunction and also develop gestational diabetes; this may continue from one generation to another, providing a case of adulthood disease of fetal origin.

**Some other clinical studies**

It is more or less now establish that clinically taurine has beneficial effects in elevating problems associated with major organs. In a study of diarrhea associated with diabetes (1), it was found that bile acid mal-absorption is an important cause of diarrhea in patients. Taurine containing bile salt tauroursodeoxycholate has a protective role of human liver, in harvesting and placing in cold storage before transplantation (11). Taurine has a protective role against damage of human colon cells induced by hypoxia (100). Taurine also prevents high glucose induced apoptosis of human vascular endothelium cells (101).

**Ageing, diabetes, exercise and taurine: an inner circular path towards alleviation**

Diabetes acts as a catalyst for accelerating ageing. Diabetes combined by other diseases further accelerates the ageing. Genetic, psychological, environmental and socio-economic realities are contributors in accelerating ageing. These risk factors are mostly due to change in life style, overeating, less exercise and psychological stress, which complicate the diabetes further. Regular physical activity for skeletal development is well known. Physical activity is inversely associated with morbidity and mortality of chronic diseases like cardiovascular and diabetes (102). Exercise may be the most important single lifestyle factor for both preventing and reversing insulin resistance. Physical activity can reduce insulin resistance and improve glucose intolerance in obese people (103). Physical activity ameliorates cardiovascular risk factors including insulin resistance, metabolic syndrome and diabetes (104). Exercise has a beneficial effect in the management of hypertension (105). Various studies on effects of exercise have recorded its lowering of blood pressure, and improving glucose and lipid metabolism. In all these studies, lowering
of plasma nor-epinephrine was observed. A negative correlation exits between serum taurine level and plasma nor-epinephrine by a feed back mechanism. Taurine-containing drinks have been found to stimulate cognitive performance, wellbeing and readiness potential in different studies of physical activities (106). During exercise, exchange of amino acids also takes place, which may be one of the reasons of increased serum taurine level. An increased level of plasma taurine was also recorded in exercise (107). Taurine concentration declines with age, especially in spleen, kidney, eye, cerebellum and serum. However, taurine supplementation in aged rats corrected this declining trend. Dietary taurine supplementation blunted age-related declines in serum IGF - 1 and increased the serum creatinine, and blood and urinary nitrogen, providing a beneficial effect in advanced age (108). The majority of neurodegenerative diseases are the result of elevated oxidative stress, which produces ROS, and taurine has scavenging properties of ROS. Advanced age-related problems are believed to be linked with availability of glutathione (GSH), whereas taurine synthesis from cystein which is also precursor of glutathione, links taurine to glutathione (109). Taurine seems to be important in regulating immune functions where taurine containing chloramines may serve as a signal. Taurine chloramines have been shown to down regulate cytotoxic cytokines associated with host cellular damage. Taurine itself is found to attenuate neuronal injures induced by neurotoxic fragments of β amyloid.

Thus, these beneficial effects taurine in preventing ageing, as well as increased concentration of taurine through exercise, together with alleviation of insulin resistance by exercise, may provide a synergistic effect of taurine and exercise in alleviating diabetes and thereafter ageing symptoms. This interaction is represented in Fig. 3.

Taurine in pathophysiology of diabetes
Oxidative stress and endothelial dysfunction of normal signal transduction are key constituents of the diabetic pathogenesis. The higher cellular glucose level leads to enhanced osmotic pressure. Many organs like retina and kidney, do not require insulin for glucose uptake, thus glucose is converted into fructose. In this conversion, first enzyme aldose reductase converts glucose to sorbitol, thereafter sorbitol dehydrogenase changed it to fructose. However, conversion of fructose from sorbitol is a very slow process, thus most of the converted glucose just remains in the form of sorbitol and not finally changed to fructose; this leads to accumulation of sorbitol. As sorbitol cannot be transported to other cells this creates osmotic disbalance, compelling other osmolytes like taurine to deplete. The space created by depletion of osmolytes is filled by water, resulting in cell swelling and cellular dysfunction (80, 87). Besides, creating osmotic stress, sorbitol accumulation also increases the risk of formation of reactive carbonyl compounds and AGE (Advanced glycation end products) because scavenging molecules like taurine are already depleted. Hence, reduced scavenging of reactive carbonyl compounds like malondialdehyde and 4 hydroxynonenals further increased the oxidative stress resulting in more cell damage. These data further show that taurine acts as osmo-regulator and as an antioxidant (101). Thus, we can conclude, diabetes mellitus is a heterogeneous pathological state with altered metabolism of macromolecules, like carbohydrate, proteins and lipids. This altered condition of metabolism is collectively termed as hyperglycemia, in which insulin production and secretion as well as action are altered. Latterly diabetes is combined with defects in other systems leading to diabetes cardio myopathy, vascular damage, visual degeneration, neurological disturbances and renal dysfunction or failure, however taurine is reported to take part in alleviating the suffering from all the above-mentioned conditions, perhaps working in a similar manner with similar patho-physiology involving osmo-regulation and antioxidant.

Taurine: a therapeutic agent for diabetes and in its complications
The solid experimental support and successful clinical implication provide logic for taurine to be a therapeutic agent. The maintenance of taurine's therapeutic actions. As supply of taurine by biosynthesis is not sufficient and with time, it declines, thus exogenous supply of taurine is the only alternative. Such a supply can be granted through dietary sources only and in such situation, intercellular taurine transport must be efficient. Taurine transporters must be very active and so the renal re-absorption system of taurine must be in good working condition, and situation arising for taurine depletion like sorbitol accumulation must be closely observed. Taurine is also important for bile salt formation, which in turn facilitates in improving cholesterol and lipid metabolism, hence ameliorating some of the complications of diabetes. Taurine can be used along with other molecules such as, creatine, betaine, cystein and myoinositol for therapeutic applications. To make sure taurine functions efficiently as osmolyte and scavenging free radicals, attempts must be made to minimize sorbitol synthesis by inhibiting aldose reductase,
and this will greatly reduce sorbitol accumulation, resulting in less depletion of taurine, hence ensuring taurine osmo-regulation at maximum and thus minimizing the cellular dysfunction. Recent studies show that 3000 mg/day taurine intake increased the whole blood taurine in patients with type II diabetes (110).

_Taurine analogues: A new hope to look beyond sulfonyl ureas and others_

The therapeutic management of type-2 diabetes also requires other than insulin therapy. The readily available agents for such are sulfonyl ureas. Sulfonyl ureas are a class of compounds related to sulfonamide drugs containing a moiety of \((-\text{SO}_2-\text{NH-CO-NH}-)\). Although sulfonyl ureas are the most effective agents so far, and in use for some time, the problems associated with sulfonyl urea therapy are increasing: hypoglycemia, interaction with other drugs and various kinds of gastrointestinal problems like nausea, vomiting and non-specific abdominal disorders. The recently introduced thiazolidinedions are also associated with several complications (15).

The decreasing effectiveness and increase in complications with these drugs have been greatly influenced by rapid environmental changes and genetic factors, and hence new therapeutics are urgently needed (16). Since taurine has been clinically very successful in hyperglycemia as well as in several complications of diabetes, it has potential to become an effective therapeutic agent. However, taurine does possess problems due to its strong zwitterionic nature, which makes it almost lipophobic, which limits its permeability to cross blood brain barrier, resulting in a poor rate of absorption, and a long half-life period with slow turnover rate. These limitations restrict taurine to become an ideal agent and need to develop some structural analogues of taurine while preserving the core structural molecule of taurine intact. Such analogues may constitute a new class of anti-diabetic agents and may replace sulfonyl ureas. Taurine analogues are in use for anti-cancer, anti-alcohol and anti-convulsant. These may provide foundation for further research in this area (17, 111, 112).

**CONCLUSION**

A high percentage of society is affected by diabetes. The number of deaths due to diabetes and related problems are surpassing the deaths due to deadly diseases such as cancer. Such increase in numbers is mainly due to the rapid change in way of living, environmental impact and genetic factors. Nutritional intervention is believed to be one of the best ways to alleviate diabetes. As taurine is now known to participate in alleviating diabetes through various of ways via osmo-regulation, scavenging of reactive carbonyl compounds and modulating the ion influx, this necessitates a continuous un-interpreted adequate supply of taurine as taurine concentration is altered in diabetes. This update is a holistic representation of taurine in diabetes and its actions. This update also provides qualitative and quantitative analysis of taurine in diabetes. Yet many questions are still unanswered which need greater attention like, as taurine biosynthesis is limited, making taurine availability only through external sources, it may be dietary. This demands comprehensive studies of taurine regulation, especially its transport, uptake and release in diabetes. The measurement of taurine concentration in various organs in diabetics will be an additional advantage. As taurine is provided through dietary sources, comprehensive data of taurine dietary supply and its availability in bio-system will lead to develop a guanine consideration, and formulate the optimum taurine requirement in diabetes. Though many pharmacological and physiological studies have been performed in the past, the complicated nature of diabetes requires regular update of such experiments. Taurine and insulin mutual stimulating actions are well known, however, mode of taurine involvement in insulinotropic actions requires in-depth study to enhance insulin production. It is good to use specific animal models for experimental studies and this requires more concentration on development of such species. More work should be done experimentally to prove taurine involvement in the transfer of diabetes to next generation. As taurine supplementation has provided encouraging clinical results, such studies should continue. Exercise is a natural way of alleviating diabetes and ageing; it also increases taurine concentration, and taurine has a beneficial effect in reducing ageing. A study connecting ageing, diabetes, exercise and taurine will provide very interesting results. Lastly, as taurine has some problem as an ideal therapeutic agent for diabetes, attempts must be made to develop taurine analogues overcoming the taurine limitations.

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REFERENCES


Taurine-Diabetes Interaction: from involvement to protection


A peculiar pattern of expression of the transferrin receptor (CD71) by reticulocytes in patients given recombinant human erythropoietin (rHuEPO): a novel marker for abuse in sport?

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ABSTRACT: To overcome the limitation of the currently adopted direct method to detect recombinant Human Erythropoietin (rHuEpo) abuse in sport, indirect analysis of blood parameters are increasingly used as part of the anti-doping strategies. The aim of the present work is to identify whether immunophenotype modifications on erythroid cells may be indicative of previous rHuEPO administration. The study was conducted on dialyzed patients under treatment with rHuEPO (DPT). Dialyzed patients without rHuEPO therapy (DP) and volunteer donors (H) were used as controls. The analysis of erythroid cells immunophenotype, performed using a multiparametric flow cytometry technique, showed a peculiar pattern of CD71 expression following rHuEPO treatment. In particular CD71 showed an increased expression in mature and intermediate reticulocytes and a surprisingly decreased expression in immature reticulocytes. In conclusion, the analysis of reticulocyte maturation stages with TO/CD71 double staining may be considered as a valid alternative indirect method for the detection of rHuEPO abuse.

KEY WORDS: Erythropoietin, Blood doping, Immunophenotype, Reticulocyte

INTRODUCTION

The isolation of erythropoietin (EPO) in 1977 paved the way for cloning the gene and producing recombinant human erythropoietin (rHuEPO) (1-3). The gene for EPO encodes a protein precursor of 193 amino acids, which after cleavage of 27 amino acids, glycosilation of four amino acids and removal of the C-terminal arginine yields the final circulating EPO molecule with 165 amino acids. rHuEPO was launched as a pharmaceutical product in 1985 and a longer-acting erythropoietin analogue, darbepoietin (dEPO), also known as novel erythropoiesis-stimulating protein (NESP), was launched in 2001 (4).

EPO acts on erythroid progenitor cells in the bone marrow (5). The earliest committed erythroid progenitor cells are called Burst-Forming Unit-Erythroid (BFU-Es) and they are characterized by a high proliferative potential and a low number of EPO receptors (EPO-R). During maturation toward Colony-Forming Unit-Erythroid (CFU-E), they gradually lose their proliferative capacity and EPO receptors increase in number. EPO acts synergistically with a number of growth factors and cytokines, including the Insulin-like Growth Factor-1 (IGF-1) to cause maturation and proliferation from the stages of BFU-E and CFU-E (6, 7).
The highest number of EPO receptors are seen on CFU-Es and pronormoblasts where EPO acts primarily reducing apoptosis (8, 9). Then, the number of EPO-R per cell gradually decreases. Reticulocytes and mature erythrocytes contain scanty, if any, EPO-Rs (8-11).

Reticulocytes in the peripheral blood normally comprise about 1% of the red cells. Their number rises when red cell production is vigorous, as after a major bleeding episode or after bone marrow regeneration following its suppression (12).

More immature reticulocytes, also called young reticulocytes, shift or stress cells, normally constitute less than five percent (5%) of the total number of reticulocytes. They contain the greatest quantity of RNA, that nucleases degrade over a period of approximately three days (13).

With regard to sports medicine, the use of rHuEPO as an ergogenic aid for the enhancement of aerobic performance is estimated to be a practice for 3 to 7% of elite endurance sport athletes (14).

The method currently accepted by the World Anti-doping Agency (WADA) to detect recombinant EPOs in urine is based on the analysis by isoelectric focusing, double blotting and chemiluminescent detection (15). Unfortunately, this method is unable to detect EPO abuse some days (2-7 depending of the dosage and the administration route) after the end of the pharmacological treatment. To overcome this limitation, several indirect methods have been proposed and developed (16, 17). In a recent meeting of medical representatives from International Sports Federations involved in the measure of blood parameters, there was general consensus that the analysis of blood parameters should be considered as a part of the anti-doping strategy because it can help to identify suspicious profiles, and at the same time may act as a strong deterrent.

Previous studies have shown that erythropoietin not only increases erythrocyte production but it is also essential for the synthesis and the correct functioning of several erythrocyte membrane proteins (18, 19).

In particular, it has been suggested that rHuEPO administration is able to modify the expression of CD35 (Complement Receptor Type I), CD59 (Complement Regulatory Proteins), CD71 (transferrin receptor) and GPA (Glicophoryn A) proteins on erythroid cells (20-24).

The purpose of this work is to evaluate the pattern of expression on reticulocytes of surface molecules which could be used as marker of previous treatment with rHuEPO. We conducted the study on dialyzed patients in treatment with rHuEPO (dialyzed patients treated, DPT), dialyzed patient without such therapy (dialyzed patients, DP) and volunteer donors as controls (healthy, H) in order to identify differences, if any, of the immunophenotype on circulating reticulocytes at different maturation stages with the use of a flow cytometry technique.

**MATERIALS AND METHODS**

**Donors**

The first group of subjects was composed of 9 patients in haemodialysis (5 females and 4 males, age range 45-64) in treatment with rHuEpo (DPT). The drug was administered three times a week by intravenous injection. The administration of rHuEPO intravenously (iv) was adopted as a Good Medical Practice subsequent to the information note from the Italian Health Minister, following a Dear Doctor Letter from Janssen-Cilag. This document recommended the route of rHuEPO administration and reported the increased risk of Pure Red Cell Aplasia (PRCA) secondary to treatment with subcutaneously (sc) rHuEPO administration as reviewed by Cournoyer et al. and Schellekens (25, 26). Moreover, some studies have reported that the iv route is as effective as the sc route (27). The dosage was adjusted according to the hemoglobin (Hb) values, with a target level of 11-12 g/dl, according to the current international guidelines on anemia treatment (rHuEPO: dose range 67-167 UI/kg, rHuEPO): dose range 42-80 UI/kg, darbepoietin alpha: 1.5 UI/kg). None of the patients received blood transfusions or other treatment known to interfere with the immune system in the three months prior to the sampling procedure. As indicated in the international guidelines, iron status parameters were periodically monitored and intravenous iron was administered in order to maintain appropriate ferritin levels (target 150-300).

The second group was composed of 10 patients in haemodialysis who attained good Hb levels (above 11 g/dl) without rHuEPO treatment (dialyzed patients, DP) (7 females and 3 males, age range 39-70). The selection of this second group aimed to match the renal disease with the DPT group in order to avoid differences due to the pathogenesis of the disease leading to chronic haemodialysis.

None of the subjects was suffering from chronic or acute infections or neoplasms during the study; all patients were treated in an out-of-hospital dialysis unit, where only patients without severe intercurrent diseases are followed.

The third group was composed by 16 healthy volunteers (H) used as controls (11 females and 5
males, age range 38-65).

In all groups, after signing the consent form, the subjects underwent venipuncture, and 4 ml of whole blood were collected into K$_2$EDTA containing tubes. The collected blood was stored at room temperature for no more than one hour and immediately treated for immunophenotype analysis.

The main demographic characteristic data, haemocromocytometric values and data regarding iron status and levels of ferritin of all subjects are summarized in Table I.

The study was designed in agreement with the declaration of Helsinki and was approved by the local Ethical Committee.

Reagents
Retic-Count (thiazole orange, TO) was purchased from Becton Dickinson (San Jose, CA). The monoclonal antibodies (MoAbs) tested on reticulocytes, all Phycoerytrin (PE)-conjugated, were: CD71, CD35, CD59, CD105 and glicophoryn A. The monoclonal antibodies tested on lymphocyte were Tri-Color® (TC) and Peridinin Chlorophyll Protein (PerCP) – conjugated and were: CD45, CD3, CD19, CD33. All MoAbs were purchased from Caltag Laboratories (San Francisco, CA, USA).

Cell preparation
The diluted 1:200 whole blood was incubated at room temperature in the dark for 20 min with mouse antiserum. Then appropriate monoclonal antibodies were added in the tube and incubated at room temperature in the dark. After washing with PBS (Phosphate Buffered Saline) and centrifugation (10 min at 700 rpm) the pellet was suspended in 1 ml of Retic-COUNT fluorescence reagent and incubated at room temperature in the dark for 30 min.

Immunophenotyping
Data acquisition was performed with FACS (Fluorescence Activated Cell Sorting) Calibur cytometer Becton Dickinson (BD, San Jose, CA, USA) using CellQuest Pro software program. A count of 100,000 erythroid cells was taken per sample. Erythroid cells were identified using forward/side scatter gating. FL1 fluorescence (green fluorescence detectable in channel 1), TO staining, was used for the separation of the reticulocytes from red blood cells. All cluster of differentiation (CD) were detectable in FL2 (red fluorescence detectable in channel 2).

For the standardized calibration of flow cytometer, normal peripheral blood lymphocytes stained with CD4FITC/CD8PE/CD3PerCP were used, PMT (photomultiplier tube) setting being checked out with calibrate beads (Becton Dickinson) in order to assess intralaboratory variability. Forward scatter (FSC), side scatter (SSC), and fluorescence data were analyzed.

Subsequently, scattergraphs TO/CD35, TO/CD59, TO/CD105, TO/GPA and TO/CD71 were analyzed in each sample. Considering that no significant differences were observed in the analyzed scattergraphs among the three groups studied (H, DP, DPT) except for the TO/CD71, the study proceeded using those two parameters (TO and CD71).

Procedures
The gate R1 in scattergraphs FSC/SSC logarithmic scale was used in order to isolate erythroid cells from the other blood cellular elements (Fig. 1). Subsequently, reticulocytes were isolated using scattergraphs TO/CD71 dot plot.

In order to easily identify the reticulocyte pattern of maturation, we decided to use the four dot plot regions, where cells localize because of their phenotype, identified on the base of TO and CD71 intensity as follows:

- **B0** - TO/CD71⁻ (negative population): erythrocytes
- **B1** - TO$^{\text{low}}$/CD71$^{\text{low}}$: mature reticulocytes
- **B2** - TO$^{\text{low}}$/CD71$^{\text{med}}$: intermediate reticulocytes
- **B3** - TO$^{\text{low}}$/CD71$^{\text{high}}$: immature reticulocytes

Those dot plot regions were used with the philosophy of empty boxes as previously described (28, 29).

We focused the analysis on CD71+ cells. Even if the small proportion of TO$^{\text{low}}$ cells maybe considered as reticulocytes because of the presence of nucleotides (A0 in Fig. 1), we decided not to include them in the so-called population of “functional reticulocytes” characterized by the presence of the transferring receptor.

Following the analysis of the whole reticulocyte population for the count of reticulocyte percentage, in each sample other three different analysis were performed acquiring 5,000 cells in B1, B2 and B3 gate respectively for the quantitative analysis of the TO and CD71 fluorescence intensity.

The mean antigen expression together with the intensity of TO staining were then evaluated in each box.

Considering that a small population of TO$^{\text{med high}}$/CD71$^{\text{low med}}$ cells was found in all of the analyzed samples, a reverse gating of those cells was performed in order to understand where they localize within the whole blood population in the scattergraphs FSC/SSC (Fig. 1). Subsequently, CD45, CD3, CD19 and CD33 antigens were
A peculiar pattern of expression of the transferrin receptor (CD71)

**TABLE I - SUBJECTS RECRUITED FOR THE STUDY. IT IS REPRESENTED THE MAIN DEMOGRAPHIC CHARACTERISTIC DATA, HAEMOCROMOCYTOMETRIC VALUES AND DATA REGARDING IRON STATUS AND LEVELS OF FERRITIN AT THE MOMENT OF THE STUDY. THE KIND OF PHARMACOLOGICAL EPOEITIN AND THE MEAN OF DOSE ADMINISTERED ARE LISTED FOR DPT PATIENTS.**

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<th>SUBJECTS</th>
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<th>RBC* 10^12/L</th>
<th>HBG* g/dl</th>
<th>HCT* %</th>
<th>IRON_g/dl</th>
<th>FERRITIN ng/ml</th>
<th>PHARMACOLOGICAL EPOEITIN</th>
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* erythrocyte RBC, hemoglobin HBG, hematocrit HCT; † (DPT) dialyzed patients in treatment with rHuEPO, (DP) dialyzed patients without treatment, (H) healthy donors

**TABLE II - MULTIPARAMETER FLOW CYTOMETRY ANALYSIS OF RETICULOCYTES IN HEALTHY DONORS (H), DIALYZED PATIENTS WITHOUT TREATMENT (DP) AND DIALYZED PATIENTS IN TREATMENT WITH RHUEPO GROUPS (DPT). VALUE ARE SHOWN AS MEAN ± SD.**

<table>
<thead>
<tr>
<th>Box</th>
<th>H</th>
<th>DP</th>
<th>DPT</th>
<th>p value H vs DP</th>
<th>p value DP vs DPT</th>
<th>H</th>
<th>DP</th>
<th>DPT</th>
<th>p value H vs DP</th>
<th>p value DP vs DPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>36.38 ± 6.41</td>
<td>34 ± 7.64</td>
<td>25.29 ± 2.14</td>
<td>NS</td>
<td>0.009</td>
<td>18.90 ± 1.36</td>
<td>19.1 ± 1.91</td>
<td>26.4 ± 2.64</td>
<td>NS</td>
<td>0.001</td>
</tr>
<tr>
<td>B2</td>
<td>108.75 ± 22.73</td>
<td>99.3 ± 28.27</td>
<td>61 ± 12.26</td>
<td>NS</td>
<td>0.004</td>
<td>275.25 ± 41.51</td>
<td>278.2 ± 7.38</td>
<td>336.4 ± 17.13</td>
<td>NS</td>
<td>0.002</td>
</tr>
<tr>
<td>B3</td>
<td>121.88 ± 23.87</td>
<td>108.6 ± 35.01</td>
<td>101.6 ± 22.04</td>
<td>NS</td>
<td>NS</td>
<td>3212 ± 871</td>
<td>3164 ± 909</td>
<td>2245 ± 445</td>
<td>NS</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Student's test
analyzed in this population in order to avoid any white blood cell or platelet contamination of the reticulocyte analyzed population.

**Statistics**

Statistical analysis was performed using the Primer of Biostatistics Package (McGraw-Hill, New York NY). Statistical comparisons were performed using Student’s t-test (considering significative a p value < 0.05).

**RESULTS**

**Phenotypic pattern**

Initial investigations were directed to identify the best labeling procedure in order to optimize the reticulocyte isolation and their identification using flow cytometry. In contrast to the common procedure of reticulocyte counting with a single stain we used a double direct staining TO (FL1) and CD71 PE-conjugated (FL2) creating a dot plot region TO/CD71 for a more precise isolation of reticulocytes from red blood cells and for a better and easier identification of the pattern of maturation.

A small population of TO

\[
\text{med high /CD71}\text{low med}
\]

was found in all samples. The analysis of those cells showed that they expressed CD45 and CD3 (A1 in Fig. 1). Therefore, this population of small lymphocytes was excluded from any further analysis. The scattergraph TO/CD71 designed, in each group, a characteristic pattern that we defined “cornucopia” easily defining the pattern of reticulocyte maturation as shown in Fig. 2.

The mean expression of TO and CD71 analyzed in each box referred to groups H, DP and DPT are summarized in Table II. Obviously, patients treated with rHuEPO had a higher number of reticulocytes when compared to H and DP groups.

In order to improve the study of reticulocyte maturation we use three boxes where cells localize according to their stage of maturation. These stages were identified on the base of reticulocyte phenotypes (namely the TO and CD71 intensity). Boxes were indicated as B1 (mature reticulocytes), B2 (intermediate reticulocytes) and B3 (immature reticulocytes).

As expected, treatment with rHuEPO increased the percentage of intermediate (B2) (p=0.006) and immature (B3) (p=0.011) reticulocytes (Table II).

CD71 expression showed a highly significant increase in values in B1 (p=0.001) and B2 (p=0.002) when comparing DP to DPT respectively (Table II). Surprisingly, CD71 expression in B3 was significantly lower in DPT when compared to DP (p=0.026).

To intensity was significantly lower in B1 and B2 boxes in DPT when compared to both DP and H. No significant difference were seen in B3.

Moreover, the mean of intensity of CD71 and TO of each subject were analyzed in combination and subsequently plotted in a bidimensional graphic in which the separation among DPT patients from the other subjects was evident even more clearly in particular in boxes B1 and B2 (Fig. 3). This model rendered highly significative the co-expression of TO and CD71 as a marker of previous rHuEPO administration and in particular the lower expression of CD71 in B3 in DPT seems to be a characteristic of previous rHuEPO administration even considering that no differences had been observed in patients treated with different pharmacological preparations (epoietin and darbepoietin).

**DISCUSSION**

Anaemia, in the setting of chronic kidney disease (CKD), is a well-recognized multifactorial phenomenon which is associated with decreased renal function and related deficiency of erythropoietin as well as chronic inflammatory status. In our study, with the aim of reducing bias, we selected only rHuEPO responder patients in which we may suppose that the emopoiesis is less affected by extrarenal disturbances. Moreover, we analyzed two control groups of subjects:

1. Dialyzed patients without rHuEPO therapy, matched for age, sex and disease causing renal disfunction.

2. Healthy volunteers matched for age and sex.

In our study no differences were observed between dialyzed patients without rHuEPO treatment (DP) and healthy subjects (H) when considering all analyzed parameters. Only in dialyzed patients in therapy with rHuEPO (DPT) a different biological behaviour was evident. Therefore, we may suppose that the observed phenomenon depends only on rHuEPO administration and it is not caused by renal disease and/or chronic haemodialysis.

To obtain an effective deterrent to blood doping, it is obvious that reliable methods are needed to detect the haematological modifications caused by the adopted illegal methods. It is agreed that a major limitation of the urine-based method for evaluating rHuEPO is attributable to its pharmacodynamic characteristics. It seems unlikely, moreover, that...
there will be major improvements in the isoelectric focusing and double blotting method ability in the detection of rHuEPO misuse even in light of the recent introduction of the use of rHuEPO microdoses as maintenance treatment. Most importantly, in the near future we will face the introduction of novel agents that reportedly produce meaningful and lasting increases in hemoglobin levels, such as EPO receptor agonists or activator of the hypoxia inducible factor (HIF)-mediated endogenous EPO synthesis (30, 31). As a logical consequence, indirect methods for assessing previous rHuEPO use have been developed. Haematological indices (number of red blood cells, blood haemoglobin concentration, hematocrit, reticulocyte count, number of hypochromic macrocytes) and

![Fig. 1 - The gate R1 in scattergraphs FSC/SSC logarithmic scale was used for the isolation of erythroid cells. The four boxes identified reticulocytes stages of maturation as follows: B0 - TO/CD71 (negative population): erythrocytes B1 - TO\textsuperscript{low}/CD71\textsuperscript{low}: mature reticulocytes B2 - TO\textsuperscript{low}/CD71\textsuperscript{med}: intermediate reticulocytes B3 - TO\textsuperscript{low}/CD71\textsuperscript{high}: immature reticulocytes Box A1 includes the population of CD45+/CD3+ small lymphocytes.]

![Fig. 2 - Comparison of TO7CD71 scattergraphs in the three analyzed populations. DPT: dialyzed patients under treatment with rHuEPO; DP: dialyzed patients without rHuEPO therapy; H: volunteer donors.]

biochemical parameters (serum EPO and soluble transferrin receptor concentrations) are widely used for this purpose and mathematical models simultaneously utilising many biomarkers have been developed (16).

In literature it has been suggested that rHuEPO administration is able to modify the expression of several cell surface proteins of the erythroid lineage (20-24), as well as the induction of particular erythroid gene profiles (32). Unfortunately, conflicting results arose from those studies. Therefore, the main purpose of the present study is to investigate whether rHuEPO administration is able to induce modifications on cell surface antigen expression in circulating reticulocytes.

The analysis of the cell surface antigens performed on our sample did not confirm that the administration of rHuEPO modifies the reticulocyte expression of CD35, CD59 and GPA, as previously suggested in literature (20-24).

The presence of residual nuclear organelles differentiates reticulocytes from mature erythrocytes (33). The organelles contain ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). TO (thiazole orange) binds to both RNA and DNA, forming a fluorescent nucleotide-reagent complex which exhibits an absorption band at 475 nm (blue light) and fluorescence emission band at 530 nm (green light). This property is commonly used in flow cytometry for reticulocytes isolation.

The transferrin receptor is a transmembrane glycoprotein involved in iron metabolism and specifically in the cellular uptake of transferrin during erythrocyte ontogeny. CD 71 begins to be expressed at the BFU-E stage and disappears at the late reticulocyte stage (34). Its persistent presence, after release from the bone marrow, defines an early population of reticulocytes (35).

Usually the reticulocyte analysis and the immature reticulocyte fraction calculation (IRF) is performed using a single staining procedure. In the present study, double staining TO/CD71 allowed a better definition of the pattern of reticulocyte maturation leading to a more precise and easier identification of the different stages of maturation. This evidence allows us to suggest this double staining as a valid method for the study of the different reticulocyte sub-populations.

Fig. 3 - The intensity mean of CD71 and intensity mean of TO were analyzed in combination and subsequently plotted in a bidimensional graphic in which the separation among DPT from the other groups was evident. The bidimensional analysis of the co-expression of TO and CD71 showed a significant separation between the treated and untreated patients more evident in B1 and B2.
As expected rHuEPO led to an increase in the more immature reticulocytes as an effect of the pharmacologically increased hemopoiesis.

The expression of CD71 underwent significant modifications related to treatment with rHuEPO. Interestingly, in all of the treated patients an increased expression of CD71 in mature and intermediate reticulocyte subpopulations was observed, suggesting that treatment with rHuEPO is able to modify the phenotype of those cells.

Even more interestingly, in all of the rHuEPO treated patients, although bone marrow stimulation produced an increase of the immature reticulocytes, we observed a significant reduction of CD71 expression in comparison to the samples drawn from the rHuEPO untreated patients and healthy donors. The reduction of CD71 in B3 could be explained by a shedding of transferrin receptor. In fact, some studies reported that the shedding of transferrin receptor is regulated by iron deficiency and erythropoietin stimulation (36, 37). Even if the value of soluble transferrin receptor observed in our sample contrast this hypothesis, we do not have an obvious explanation for this phenomenon which is paradoxical in the sense that an increase red cell production with an increased release of immature reticulocytes is coupled with a decrease transferrin receptor expression. As a matter of fact, it is known that CD71 is usually highly expressed on immature reticulocytes and its expression decreases during the way approaching the erythrocyte status.

The reduced TO intensity of mature and intermediate reticulocytes coupled with increased CD71 expression observed in our treated patients has relevance also to the calculation of reticulocyte IRF. In fact, cells with a TO low intensity are considered mature with the single staining procedure while they can be attributed to intermediate with the double staining procedure because of their CD71 expression.

In conclusion, TO/CD71 double staining procedure should be considered not only as an improvement in the study of the reticulocyte pattern of maturation but also a valuable method for the detection of previous rHuEPO administration.

When considering that all of the evaluated patients in rHuEPO treatment did show the same behaviour, a prospective, longitudinal protocol of extension of this pilot study needs consideration. This method could represent a novel approach in the fight against doping for revealing erythroid cell modifications related to the abuse of rHuEPO by athletes. Moreover, those indirect parameters could be added to other haematological and biochemical indices in a multiparametric analyses, in order to improve the sensibility and specificity of the tests actually used to screen the abuse of haematological growth factors.

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A peculiar pattern of expression of the transferrin receptor (CD71)


iNOS activity in the aged rat liver tissue


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ABSTRACT: Free radical damage to many cellular components has been proposed as the main mechanism underlying the aging process. In the liver, NO can be generated by iNOS, but also by the constitutively expressed endothelial NOS (eNOS). iNOS enzyme appears to be expressed in liver disease such as cirrhosis and fulminant hepatitis, while the eNOS is expressed in physiological conditions. Ten young and ten old Wistar rats were sacrificed and their livers were excised. Liver sections were incubated with an anti-iNOS antibody of rabbit origin. RT-PCR and Western blot analysis were performed and nitric oxide activity was calculated. A significant increase of iNOS immunoreactivity was seen in the aged liver sections versus young liver sections. iNOS protein is expressed in greater quantities in the aged group, compared to the young group. In this study we show, for the first time, that aging in the rat liver is accompanied by a spontaneous induction of iNOS mRNA, high levels of iNOS protein and immunohistochemistry/image analysis.

KEY WORDS: Nitric oxide, iNOS, Lung cancer, Cytokines, Inflammation, Apoptosis

INTRODUCTION

Aging is the accumulation of changes responsible for the sequential alterations that accompany advancing age and the associated progressive increases in the possibility of disease and death. These changes may be attributed to disease, environment, immune dysfunction, and to an inborn process—the aging process. This produces aging changes at an apparently unalterable and exponentially increasing rate with advancing age. The cumulative damage caused by free radicals to many cellular components has been proposed as the main mechanism underlying the aging process (1). Nitric oxide (NO) is considered to be one of the major mediators of physiological, and pathophysiological processes. Nitric oxide is a free radical gas produced by the conversion of L-arginine to L-citrulline, catalyzed by nitric oxide synthase (NOS), in the presence of O₂ (2). In humans, three genes located on separate chromosomes, encode distinct isoforms of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) (3). Although iNOS induction is a well-known mechanism of immune defense triggered by cytokines against infection, cancer and during inflammation, it has recently been shown that this process may occur without an obvious underlying pathological condition in aging, leading to an elevated constant level of NO in the affected tissues (4-7).

iNOS is one of the three isoforms of NOS expressed in murine macrophages, hepatocytes and Kupffer cells which can be activated by many stimuli including mycobacteria, endotoxin, and cytokines, responsible for a greater NO production upon induction (8). NO can act in both anti-inflammatory and pro-inflammatory manner by modulating NF-kB activity and transcription of iNOS (9-12) and also have bactericidal and cytotoxic activities in murine macrophages (13, 14). In the liver, NO can be generated by iNOS, but also by the constitutively expressed endothelial NOS (eNOS). In human tissues, the iNOS enzyme appears to be expressed in liver disease such as cirrhosis and fulminant hepatitis, while the eNOS is expressed in physiological conditions (15, 16).

Animal studies have shown that depending on experimental models of liver injury, NO derived from iNOS can either prevent or mediate liver damage.
iNOS activity in the aged rat liver tissue

(17-22), therefore, the role of NO from iNOS is unclear. Accurate assessment of NO production is very difficult (23). In the present work, we have chosen some of the best techniques by RT-PCR, Western blot, and L-citruline assay to assess NO production. We investigated whether iNOS is induced spontaneously in the liver of the rat during aging by mRNA and protein iNOS expression and localization an NO production in young and aging rats.

**Materials and methods**

**Animals**

Ten young male Wistar rats (2 month old) and ten aged male Wistar rats (25 month old)) were used in this study. They were bred in infection-free environment and fed a low fat diet. Exercise was limited since they were caged. The animals were anaesthetized with Nembutal (30 mg/Kg i.p.) and were sacrificed after administration of heparin (1000 UI/kg) into the femoral vein. The livers were excised, immersed in liquid nitrogen and kept at –80 °C.

**iNOS Immunohistochemistry**

Tissue sections (10 μm thickness) were cut with a cryo-microtome (Reichert-Jung “Frigo cut 2800”, Leica, Germany), mounted on microscope slides, fixed by immersion in acetone at 4°C for 5 min and air-dried. Slides (5 for each rat) were treated with 5% normal goat serum, 0.1% bovine serum albumin and 0.1% Tween 20, in PBS for 30 min at room temperature. The slides were incubated for 30 min. with a 1:100 in PBS anti-iNOS antibody of rabbit origin (Affinity, GB) which was diluted and applied at room temperature. Afterwards, the slides were washed in PBS three times for 5 min each, and then incubated for 30 min with biotinylated secondary antibody.

They were then washed in PBS three times for 5 min. and incubated for 30 min with Avidin Biotin (AB), washed in PBS three times for 5 min and incubated with peroxidase substrate for 5 min, finally washed in tap water for 5 min and dehydrated (Rabbit ABC Staining System. Santa Cruz Biotech, Inc. Santa Cruz, California). Slides from immunohistochemical reaction were mounted using glycerol, cover-slipped, and photographed through an optical microscope (Leica-Germany) (24).

**Semi-quantitative reverse transcription-polymerase chain reaction for iNOS**

Semi-quantitative reverse-transcribed polymerase chain reaction (RT-PCR) was used to determine mRNA levels of the iNOS in rat liver tissue. Total RNA was extracted using 1 ml/g ULTRASPEC-RNA (Biotech, Lab., Inc. Huston, TX, USA), as recommended by the manufacturer. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding RNA (1μg) to a mixture containing 1 mM deoxy-nucleoside- triphosphates (dNTP), 1U/μl RNase inhibitor, 2.5 U/μl moloney murine leukemia virus reverse transcriptase, 2.5 μM oligo-dt, 5 mM MgCl2, 10x PCR buffer in a final volume of 20 μl. Reverse transcription was performed at 42°C for 1 h followed by heat inactivation of reverse transcriptase at 92°C for 10 min. 18S was amplified from the same amount of RNA to correct for variation of different samples. PCR amplification was performed using a Programmable Thermal Controller (MJ Research, Inc. Massachusetts, USA). The PCR solution contained 10 μl of first-strand cDNA, 4 μl 10x PCR buffer, 2 mM MgCl2. The following primer pairs (MWG-Biotech AG, ITALY) were used: sense 5’-CGT AAA GAC CTC TAT GCC AA-3’ and antisense 5’-AGC CAT GCC AAA TGT CTC AT-3’ for iNOS and 18S primers, 0.15 mM of both sense 5’-TAC GGA GCA GCA AAT CCA C-3’ and antisense 5’-GAT CAA AGG ACT GCA GCC TG-3’, 2U Thermophylus Aquaticus (Taq) DNA polymerase (Celbio, Milan, Italy), and water to a final volume of 50 μl. These samples were overlaid with mineral oil and subjected to 35 cycles at 95°C for 60s, 60°C for 60s, and to one cycle at 72°C for 7 min. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UV light. Bands on the gel were scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

**Western blot analysis for iNOS**

Determination of iNOS protein was performed in three series of protein extracts by Western blotting (WB). 50 μg cytoplasmatic proteins, quantified, by spectrophotometric assay (HP 8452A, California, USA) using Lowry method, from rat liver were separated by electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Bio-RAD), Hercules, California) and transferred at 4°C to nitrocellulose membrane (Bio-RAD, Hercules, California) in glycine-methanol buffer. Nitrocelluloses was then blocked in Tris-Buffered Saline (TBS)-milk and incubated, overnight, with an anti-iNOS primary antibody (1:1000) (Santa Cruz Biotech, Inc. Santa Cruz, California). The nitrocelluloses were then washed in TBS, incubated with a secondary antibody conjugated with alkaline phosphatase for 2 hr, washed again, and developed in an alkaline buffer with nitrobluetetrazolium (NBT) as substrate (Alkaline Phosphatase Conjugate Substrate Kit, BIO-RAD, Hercules, California). β-actin (Sigma, 1/10.000) was used as an internal
standard. The densitometric analysis of western blots was performed using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

**Citricline synthesis (nitric oxide activity)**

The measure of the conversion of L-arginine to L-citrulline is a standard assay method currently used to quantitative NOS activity. Briefly, 1 µl of radioactive arginine, L-(2,3,4,5)-[^3]H]Arginine Monohydrochloride (1µCi/µl; Amersham, Arlington Heights, Illinois, USA), 5 µl NADPH 10 mM, 25µl reaction buffer 2X (50 mM tris-HCl (pH7.4), 5µM BH4, 2µM FAD, 2µM FAM) were added to each tissue homogenate samples (10 µl) and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 µl of stop-buffer (50 mM HEPES, pH 5.5, 5 mM EDTA) and added to the equilibrated resin into each sample. The equilibrated resin bound unreacted arginine. After centrifugation, the radioactivity corresponding to L-[^3]H]-citrulline was measured with liquid scintillation spectrometry. Calcium was omitted from these incubations to favour the determination of the calcium independent iNOS isoform. In the incubation medium for iNOS measurement, the calcium in the sample homogenates was removed with EDTA to prevent the measurement of any constitutive NOS activity. Control incubations were done in the absence of NADPH. The stopping buffer also contained L-citruline to block any further conversion from L-argenine.

**Image processing and analysis system**

The densitometric analysis was performed by using a LEICA Quantimet 500 plus (LEICA Cambridge Ltd, Cambridge, England) determining the change in Integrated Optical Density (I.O.D.) using ISO transmission density standard Kodak CAT 152-3406 (Eastman Kodak Company, Rochester, USA).

**Statistical analysis**

The results were expressed as mean ± SD. Statistical analysis was performed using the analysis of variance (ANOVA), p<0.05 is considered statistically significant.

**RESULTS**

**iNOS immunohistochemistry**

To investigate the functions of iNOS in liver tissue of young and aged rats, we localized it with anti-iNOS antibody (Fig.1). A significant increase of iNOS immunoreactivity is present in panel B (Aged liver) versus panel A (Young liver). The stained kidney structures I.O.D. values were quantified and are shown in Table I.

**Fig. 1** - To investigate the presence of iNOS in the liver tissue of young and aged rats, we localized it with anti-iNOS antibody. A significant increase of iNOS immunoreactivity is present in panel B (Aged liver, darker picture) versus panel A (Young liver, lighter picture).

**Fig. 2** - RT-PCR analysis. The presence of iNOS in the young rat liver is barely detectable, however a marked increase of iNOS was found in aged rat liver tissue. 18S (488 bp) is an internal standard.
iNOS activity in the aged rat liver tissue

**iNOS- rtPCR analysis**

To determine whether aging is associated with a spontaneous induction of iNOS in liver tissue, total RNA was isolated from the liver from 25 month old (aged) and 2 month old (young) Wistar rats and an RT-PCR was performed. Fig. 2 shows a significantly greater expression of iNOS mRNA in the liver of aged versus young rats.

**iNOS Western blot analysis**

To investigate the amounts of iNOS in liver of young and aged rats, their protein levels were detected. As shown in the representative experiment of separate experiment (n=3) (Fig. 3). Western blot analysis demonstrates that iNOS protein is expressed in greater quantities in the aged group, compared to the young group.

**Inducible nitric oxide synthase activity**

To assess whether the observed increase in iNOS expression in the livers of aged rats corresponded to the enhanced enzyme activity, we evaluated NOS enzymatic activity in both tissue groups. As shown in Figure 4, in liver homogenate of aged rat, basal L-[3H]-citrulline synthesised from L-[3H]-arginine was 0.022 ± 0.004 pmoles/min/mg total protein. This was significantly greater than the conversion rate observed in young rat, which was 0.008 ± 0.002 pmoles/min/mg total protein (P<0.001 vs. Young).

**DISCUSSION**

In this study we found, for the first time, that aged (25 month old) rat liver tissue expressed large amounts of iNOS through the spontaneous induction of iNOS mRNA and high levels of iNOS protein, compared to young (2 month old) rat liver tissue. This was clearly analyzed by four different methods. Biological aging is a complex process featuring species- and tissue-specific rates and molecular mechanisms of age-related physiological and molecular changes (25).

Evidence has been obtained that biological aging is a function of several closely interrelated parameters such as the metabolic rate, caloric intake, genetics, lifestyle and environmental factors (216-32). Aging is associated with increasing steady-state levels of oxidatively modified biomolecules as a result of free radical reaction (33).

Liver disease is associated with oxidative stress (34). There is evidence of oxidative stress in liver disease as increased plasma and tissue levels of markers of lipid peroxidation (LOOH- and MDA) and reduction of hepatic and plasma antioxidant content. (35). Macrophages are involved in this process since they generate ROS e RNOS, which are stimuli for fibrinogenesis.

NO is currently considered as a fundamental intercellular and intracellular signalling molecule essential for the maintenance of homeostasis, which is synthesized in the liver by different NOS

**TABLE I - STAINED KIDNEY STRUCTURE I.O.D. VALUES**

<table>
<thead>
<tr>
<th></th>
<th>iNOS</th>
<th>young</th>
<th>aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immuno-histochemistry</td>
<td>1.2 ± 0.5</td>
<td>2.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Western blot</td>
<td>0.09±0.3</td>
<td>3.1±0.5</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>0.4 ± 0.2</td>
<td>2.6 ± 0.5</td>
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</table>
isoforms, but iNOS is responsible for a greater NO production upon induction (36-37). High levels of NO are responsible for most of the pathophysiological manifestations, the situation is aggravated due to the coupling of NO and O2- to form ONOO- that is a reactive RNOS. RNS inhibit the respiratory chain complexes leading to a further increase in O2- production, oxidative damage, and apoptosis.

Our results indicate that aging in the rat liver is accompanied by a spontaneous induction of iNOS mRNA as seen by RT-PCR and of higher levels of iNOS protein by both Western blot and immunohistochemistry/image analysis. We also evaluated the activities of inducible NOS isoforms using L-(3H)-arginine as a substrate, thus calculating the actual production of NO. The measurement of iNOS mRNA and protein associated with L-citruline assay, when taken together are very powerful assays; however, it is interesting that with lower iNOS activity seen in young rats are not the result of an increase in inhibition of iNOS in the early years (data not shown). We observed that in the old rat liver, the production of NO also increased. This may suggest that in the ageing rat there is an increase of anion superoxide that stimulates iNOS gene transcription through the activation of NF-kB responsive elements in the iNOS promoter (38). The increase in NO may be preventive rather than deleterious, aimed at destroying reactive oxygen species (ROS). These compounds are inducers of apoptosis, and their accumulation is linked to the aging process (39). Low concentrations of NO appear to protect cells from apoptotic cell death (40) so that the levels of NO, as well as their balance with ROS levels, would ultimately dictate the degree of cell damage, thus confirming the dual role of NO (41-43). However, to determine whether this increased NO is cytotoxic or whether it is a preventive physiological mechanism in the aging process, other study is needed.

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Comparative in vitro studies on the fibrogenic effects of two samples of silica on epithelial bronchial cells

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ABSTRACT: The small dimension and particle shape of silica in gypsum used to prepare moulds for lost wax casting might be responsible for the high prevalence of silicosis in gold jewellery. To test this hypothesis, human pulmonary epithelial cell (BEAS-2B) cultures were exposed to two samples of silica with different crystal micro-morphologies: Silica Powder (Silica P) which is used in casting gold jewellery, and no powder Silica (Silica F). Extracellular matrix (ECM) production was evaluated using radio-labelled precursors and quantified by RT-PCR analysis. Expression of basic fibroblast growth factor (FGF2) and its receptor (FGFR2) was also evaluated. The results demonstrated Silica P particles had a very fine lamellar crystalline structure while Silica F was characterized by larger rounded crystals. Silica P stimulated collagen production significantly more than Silica F and down-regulated laminin and metalloprotease expression. Both silica samples down-regulated FGF2 but only Silica F enhanced FGF2 receptor expression. In conclusion each Silica sample promoted a profibrotic lung microenvironment in a different manner and also elicited different FGF2 signalling pathways. The data confirm that different micromorphology of Silica particles affects the fibrogenic potential and the molecular mechanisms of dust pathogenicity.

KEY WORDS: Silica, Silicosis, Micromorphology, Extracellular Matrix, Basic Fibroblast Growth Factor, Epithelial Bronchial Cells

INTRODUCTION

Occupational inhalation of silica dust continues to be a major cause of silicosis (1). The composition, crystal micro-morphology and a small dimension of Silica particles could be responsible for the high prevalence of silicosis that was recently found among goldsmith preparing moulds for so-called lost-wax casting (2). Chronic pulmonary fibrosis involves excess ECM production, changes in matrix metalloproteinases (MMPs) degradation (3-6) and release of inflammatory cytokines (7-9). To determine whether the physical properties of silica modulate the molecular mechanisms of dust pathogenicity, we designed an in vitro experiment using a human bronchial epithelial cell line (BEAS-2B cells) that was exposed to powder silica particles used in lost-wax casting or no powder silica whose fibrogenic effects had been demonstrated in our laboratory (10-13). The morphology of both samples of silica particles and their internalization into cells was observed by scanning and electron microscopy. As parameters of fibrotic responses we studied collagen neosynthesis with radiolabeled precursors and performed quantitative polymerase chain reaction (PCR) analysis to determine mRNA levels of ECM components involved in lung...
microenvironment such as laminin, collagens (type IV and V), proteoglycans (decorin and betaglycan) and metalloprotease (MMP2) (14-17). Finally, to postulate a fibroblast growth factor (FGF2) autocrine mechanism that could mediate the effects of Silica on matrix production, expression of FGF2 and its receptor (FGFR2) was also evaluated. Localized in alveolar epithelial cells, alveolar macrophages, and in the bronchial epithelium, FGF2 as one of the mediators that regulate cell growth, differentiation and signalling, plays, in fact, a key role in tissue repair, in the inflammatory process and in lung fibrosis (10).

**MATERIALS AND METHODS**

**Cell culture**

BEAS-2B cells derived from normal human bronchial epithelium and immortalized by adenovirus 12-SV 40 hybrid virus transfection (18) were obtained from the American Type Culture Collection (ATCC, Rockville, MD CRL-9609).

Subconfluent cells were maintained for 48 h at 37°C and 5% CO$_2$ in BEGM medium (Cambrex, Bio Science Walkersville, MD USA) with or without the sample of Silica used for casting gold jewellery, named in our experiments Silica P (Astro-Vest Investment, Ransom & Randolph, Ohio US), or another sample of Silica with different physical and chemical properties, named in our experiments Silica F, (Sigma Chemical Company St Louis). Both the samples of Silica were used at the concentration of 50 mg/ml, (the optimal dose for observing silica effects according to Bodo et al 2001, 2003) and analysed by Scanning Electron Microscopy to determine length, size, and distribution of fibres/particles.

Silica samples were placed in Pyrex glass tubes, heated for two hours at 200° F to inactivate any endotoxin, resuspended in BEGM medium and vortexed vigorously for 2 min before use.

**Electron Microscopic Procedure**

Both silica samples were coated with gold-palladium by vacuum evaporation and viewed under a 501 Phillips Scanning Electron Microscope (SEM). To study internalization of silica into the cells, BEAS-2B were treated with or without silica P or F for 48 h in BEGM at 50 μg/ml and thin sections were obtained on a Reichert ultramicrotome, and examined through a Hitachi 800 Transmission Electron Microscope (TEM).

**Cell count**

BEAS-2B cells were treated as above, sedimented by centrifugation at 2,000 rpm and re-suspended in 1 ml of medium. Trypan Blue was added to the cell suspension to obtain a final concentration of 2 mg/ml and stained cells were counted with Burker’s camera.

**3H proline incorporation assay**

BEAS-2B were cultured as above with the addition of 50mg/ml ascorbic acid, 50μg/ml β-aminoproprionitrile fumarate and 2μCi/ml of $^3$H-labeled proline (s.a. 29Ci/mmol, Amersham Biosciences Little Chalfont, UK). Collagen was extracted as described elsewhere (19). Results are expressed as dpm/10$^6$ cells.

**Real-Time PCR Detection of mRNA levels for laminin, collagen IV, collagen V, decorin, betaglycan, MMP2, FGF2 and FGF-R2**

Total RNA was isolated from BEAS-2B cultured as above, by RNA purification kit (Versagene RNA Cell Kit, Gentra US) and quantified by reading the optical density at 260 nm. One microgram of total RNA was subjected to reverse transcription (RT) in a final volume of 50 μl. Real time PCR was performed using 1 μl of the cDNA prepared by the RT reaction.

Primers for laminin were sense 5'-GGGAATCTTGTGTTCCCCTTT-3' and reverse 5'-TTGGTTTGCAGTCACAGAGC-3'; for collagen IV were sense 5'-AAGCTGTAAGCGTTTGCGTA-3' and reverse 5'-ACTCTTTTGTGATGCACACCA-3'; for collagen V were sense 5'-AAGGGGACCTACATGTTCTGG-3' and reverse 5'-ATAGGGGAGGGCTAAAAAGG-3'; for decorin were sense 5'-GGCCACTATCATCCTCCTT-3 and reverse 5'-TTGTGGTTTTGACAGGGCTTACG-3'; for betaglycan were sense 5'-CCTGTACATCCAGTACAACT-3' and reverse 5'-ATCACCTGACTCCAGATCTTC-3'; for MMP-2 were sense 5'-TGAGCCTTACAGGACGTCAGTC-3' and reverse 5'-ATACTTCAACGAGCACTGTG-3'; for FGF2 were sense 5'-AGTGTGTGCTAATGGTACC-3' and reverse 5'-AAGATAGCTTCTGCCCAGG-3'; for FGFR2 were sense 5'-GAGTGATGTCTGGTCCTTCG-3' and reverse 5'-GCTTATCCATTCTGTGTCCT-3'; for b-actin were sense 5'-ACCTTCTACAATGAGCTGCG-3' and reverse 5'-TCCATCAAGTGCCAGTGTA-3'.

Real-time PCR was performed in an Mx3000P cycler (Stratagene, Amsterdam, Netherlands) using FAM for detection and ROX as reference dye. The mRNA level of each sample was normalized against β-actin mRNA and expressed as fold changes versus the level in untreated control cells.

**Statistical analysis**

Results reported in the figures were the mean and standard deviation (SD) of three independent
Fig. 1 - Scanning electron micrographs of Silica F particles. a) Bar: 10 µm  b) Bar: 5 µm

Fig. 2 - Scanning electron micrographs of Silica P particles. a) Bar: 20 µm  b) Bar: 5µm

Fig. 3 - Electron microscope micrographs of BEAS cells treated with Silica F (a) or Silica P (b). Uranyl acetate and lead citrate staining. Micrographs (arrows) show both silica samples are internalized by bronchial epithelial cells. a) Bar: 0.5 µm, b) Bar: 0.5 µm.
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Morphological analysis of Silica samples
Silica F: SEM showed crystals with homogeneous morphology (Fig. 1a, b), consisting of rounded aggregates with dimension of 0.5 -10 µm (approx. 80% between 1-5 µm).

Silica P: SEM showed more heterogeneous morphology with rounded aggregates of particles and aggregates of thin lamellar particles, tightly aggregated to each other with a diameter of 0.007 –0.014 µm (Fig. 2 a, b). Unlike untreated cells, TEM revealed cytoplasmic endocytosis of silica particles in both samples within single membrane vacuoles without ultrastructural cell organelle changes (Fig. 3 a, b).

Effects of Silica samples on cell count
Treatment with both silica samples decreased cell count but the effect was more marked for Silica F (data not shown) P decreased cell proliferation by –32.5% and Silica F by - 48.2%, when compared with controls.

Effects of silica samples on collagen synthesis
Collagen neosynthesis was evaluated by a radiolabeled proline incorporation study. Both samples of silica significantly increased total collagen synthesis (Fig. 4). Silica P had a

Fig. 4 - Collagen production from BEAS-2B maintained in BEGM for 48h with or without Silica P or Silica F both at the dose of 50 mg/ml. All cultures were added with [3H]-proline (2 mCi/ml). Values (mean ± SD), expressed as d.p.m./cell number x 106, were derived from three separate experiments, each in quintuplicate. Data were analyzed by analysis of variance (ANOVA). Differences vs each control: *F-test significant at 99%. Differences of Silica F vs Silica P: § F-test significant at 99%; NS not significant.
Fig. 5 - Quantitative analysis (mRNA levels) for laminin, collagen IV, collagen V, MMP2, decorin and betaglycan obtained from human bronchial normal epithelial cells, BEAS-2B. The cells were maintained in BEGM for 48h with or without Silica P or Silica F both at the dose of 50 mg/ml. The mRNA levels were quantified by real-time quantitative PCR. Values were the mean ± SD of three independent experiments each performed in triplicate. Data were analyzed by analysis of variance (ANOVA). The results were expressed as fold change in b-actin normalized mRNA values. Differences vs mRNA levels in each control: *F-test significant at 99%; NS not significant. Differences of Silica F vs Silica P: § F-test significant at 99%; §§ F-test significant at 95%; ns not significant.
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Fig. 6 - Quantitative analysis (mRNA levels) for FGF2 and FGFR2 obtained from human bronchial normal epithelial cells, BEAS-2B. The cells were maintained in BEGM for 48h with or without Silica P or Silica F both at the dose of 50 mg/ml. The mRNA levels were quantified by real-time quantitative PCR. Values were the mean ± SD of three independent experiments each performed in triplicate. Data were analyzed by analysis of variance (ANOVA). The results were expressed as fold change in b-actin normalized mRNA values. Differences vs mRNA levels in each control: *F-test significant at 99%. Differences of Silica F vs Silica P: § F-test significant at 99%; NS not significant.

significantly greater stimulating effect (+84%) than Silica F (+53%). Each Silica sample affected collagen redistribution between the intra and extra cellular compartment differently. Silica F accumulated more collagen into the cell than Silica P (+110% vs. 61%), while secreted collagen after Silica P and Silica F treatment was +89% and +41% respectively.

Effects of Silica samples on gene expression of laminin, collagen IV, collagen V, decorin, betaglycan, and MMP2

Quantitative analysis of mRNA levels (Fig. 5) demonstrated laminin expression was down-regulated after treatment with Silica P (-46.5%) and Silica F (-30.3%). Treatment with Silica P and Silica F increased the steady state of mRNA levels of collagen IV (+57 % and +70%), collagen V was strongly up-regulated (+212) after treatment with Silica P and Silica F (+35 %).

Metalloprotease levels were significantly down-regulated only by Silica P (-65%). No significant differences were induced by Silica F.

Regarding proteoglycan analysis, Silica P and Silica F significantly decreased decorin mRNA by respectively -41% and -31% (p= NS), compared with controls. Betaglycan was significantly increased by Silica F (+ 33 %) but not by Silica P.

Effects of Silica samples on FGF2 and FGF-R2 expression

Silica P and Silica F down-regulated mRNA expression for FGF2 in a similar manner (-23.4 % and -26.5 % respectively) (Fig. 6). FGFR2 expression was down-regulated by Silica P (-33.5%) and up-regulated by Silica F (+ 26%).

DISCUSSION

To test whether the physico-chemical properties and small dimension of the particles could be responsible of lung silicosis among goldsmiths (20-23), human airway epithelial cells were treated with a sample of very small diameter silica that was used in casting (Silica P) or a sample containing larger particles (Silica F). Silica P induced a significant increase in cell proliferation, enhanced collagen secretion, up-regulated collagen V m
RNA levels and decreased laminin, decorin and MMP2 expression when compared to Silica F. The decorin decrease confirms the inverse link between decorin and collagen amount (24). To investigate FGF2 autocrine mechanisms, we tested expression of FGF2 and FGFR2. Silica P decreased FGF2, FGFR2 and betaglycan mRNA levels, the latter being a low affinity FGF2 receptor (25), so we can exclude autocrine differentiation loops for FGF that underlie its fibrogenic effects. In contrast, Silica F enhanced FGFR2 and betaglycan, so we hypothesize that its biological effects are mediated by an increase in FGF2-FGFR2 binding.

The data suggest that the two silica samples induce a variety of biological responses in terms of ECM production that may differently impact upon maintenance of an intact respiratory epithelium (26-27). Since Silica effects are often related to the extent of the exposed surface (28) one may hypothesize that the very fine Silica P fibres are more dangerous because of their abundant active surface sites (Silica P external area: 200-390 m²/g, vs Silica F: 6.7-7.1 m²/g, as reported in data sheet).

Further studies are now in progress to assess overproduction of other growth factors as mediators of fibrogenic effects of Silica P in the hope of identifying possible strategies for clinical practice.

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REFERENCES

Comparative in vitro studies on the fibrogenic effects of two samples of silica on epithelial bronchial cells


Alteration of activity and survival of osteoblasts obtained from human periodontitis patients: role of TRAIL


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ABSTRACT: Periodontal disease (Pd) is characterized by extensive alveolar bone loss, that occurs as a consequence of the impairment of the normal bone remodelling. Bone remodelling is regulated by the correct balance between osteoclast and osteoblast formation and activity. Alveolar bone loss could be due to an increased bone resorption by osteoclasts or a decreased bone formation by osteoblasts (OBs) or both. Although the role played by osteoclasts in increasing bone resorption in Pd is already known, the behaviour of OBs in this disease is poorly understood. In the present study we hypothesized that activity and survival of OBs, locally present in alveolar bone of Pd patients, are altered. Thus, we studied the activity and survival of OBs obtained from alveolar bone fragments of Pd patients. The results, obtained in OBs from the patients were compared with those from OBs obtained from healthy donors. We demonstrated that OBs from Pd patients weakly express OB phenotype in respect to the control cells. In particular, the alkaline phosphatase activity and the collagen type I production, as well as the formation of mineralized nodules, typical markers of differentiated OBs, were significantly lower in Pd patients. Interestingly, we also demonstrated that OBs from the patients were more sensitive to the apoptotic effect induced by TNF-related apoptosis-inducing ligand (TRAIL). TRAIL, a member of the TNF superfamily, induces apoptosis by interacting with its death receptors, (DR4, DR5). However, its activity can be modulated by two decoy receptors, DcR1 and DcR2. Thus, the sensitiveness of TRAIL induced apoptosis is determined by the ratio of death and decoy receptor. We demonstrated that OBs from Pd patients showed an imbalanced ratio between death and decoy TRAIL receptors due to the down-regulation of DcR2 expression. Furthermore, the levels of TRAIL in the serum of the same patients were significantly higher than those detected in the controls. In conclusion, we show for the first time that the alveolar bone loss in Pd patients could be due to the increased TRAIL-mediated apoptosis of OBs.

KEY WORDS: Osteoblasts, TRAIL, Periodontitis

INTRODUCTION

Pd is the most frequent cause of tooth loss among adults. It is defined as a plaque-induced inflammation of the periodontal tissues that results in a loss of support of the affected teeth (1, 2). This process is characterized by destruction of the periodontal attachment apparatus, increased bone resorption with loss of crestal alveolar bone, apical migration of the epithelial attachment, and formation of periodontal pockets (1, 3). Bone remodelling is a coupled process determined by bone resorption and formation, thus alveolar bone loss that occurs in periodontal disease could be due to an osteoclast...
increased resorption or to a decreased osteoblast activity. Although the presence of periodontal pathogens is a prerequisite, the progression of Pd is dependent on the host response to pathogenic bacteria that colonize the tooth surface (1, 2). A number of bacterial products are capable of affecting the host response (4-8). These products initiate a local host response that involves: the generation of cytokines mostly produced by osteoblasts; the recruitment of inflammatory cells; the release of lytic enzymes; and the activation of osteoclasts (OCs) (9-11). In our previous works, we demonstrated that in peripheral blood mononuclear cells (PBMCs) obtained from periodontitis patients (Pp) the formation of OCs occurred spontaneously and it was strictly dependent on T cells (12-14). In a more recent study, it was demonstrated that also B cells have a role in increasing osteoclastogenesis process in Pp over expressing IL-7 (15-19). Moreover, it is not yet well known if osteoblast activity is locally compromised in periodontitis, causing, with a decreased bone formation, an altered bone remodelling with a consequent alveolar bone resorption (20-24).

In this work we studied osteoblast features in cells obtained from alveolar bone fragments of Pp. Considering that the compromised bone formation could be determined by increased cell mortality, we studied the eventual apoptotic effects of TRAIL on these cells.

TRAIL is a cytotoxic protein inducing apoptosis, upon binding to death domain-containing receptors DR4 and DR5. Its activity can be modulated by association with two membrane-bound decoy receptors, namely DcR1 and DcR2, lacking functional death domains and conferring TRAIL resistance on expressing cells. Thus the sensitiveness of TRAIL induced apoptosis is determined by the ratio of death and decoy receptor. TRAIL is expressed by many cells: T and B cell, dendritic cells and NK cells. Recent works demonstrated that Ob express TRAIL and its receptors but in normal conditions they are not sensitive to its apoptotic effects (25). An interesting work demonstrated that in the presence of Staphylococcus Aureus TRAIL successfully induced osteoblasts apoptosis (26) with consequent bone destruction. Moreover, the presence of S. Aureus has been demonstrated in oral cavity and periodontal pocket of a relevant percentage of patients affected by periodontal disease (27-29). The same author demonstrated that S. Aureus induces Trail expression in human osteoblasts (30). Thus, considering that in periodontal disease microbial agents, such as S. Aureus and their structure are present in periodontium, we studied whether Trail could determine apoptosis in OBs

obtained from alveolar bone of parodontopatic patients (OBs). We therefore studied OB features, their sensitiveness to TRAIL, their expression of TRAIL death and decoy receptors, and TRAIL serum levels. Osteoblasts and serum obtained from healthy donors were used as control.

**MATERIALS AND METHODS**

**Patients**

This study involved 35 Pp, recruited from the Department of Periodontology of University of Bari in the years 2003-2004, and 35 healthy donors as controls. All the patients were of Caucasian race, the mean age was 42.8 ± 15.2 years for Pp (25 men and 10 women) and 40.5 ± 5.2 years for the controls (22 men and 13 women). We selected Pp with moderate to severe generalized chronic periodontitis never treated previously. The clinical periodontal evaluation was based on the Plaque Index (PI) (31), Gingival Index (GI) (31), and radiographic and clinical evidence of alveolar bone loss. In particular, the Pp included in the study showed: PI and GI from 2 to 3 (32-34), probing pocket depth (PPD) 6.3 ±2.5mm (range 4-12 mm), probing attachment level 6.9±12.6 mm (range 5-12mm), and alveolar bone loss 50.7±18% (range 20-85%). Alveolar bone loss determination was performed using radiographic and clinical evidence of the distance between the cement enamel junction (CEJ) and the level in which periodontal ligament aspect and thickness were normal (35-36). In the controls PI and GI were 0, no evident signs of attachment loss were detected. The study was approved by the Institutional Review Board of the Department of Odontostomatology and Surgery Operative Unit of Periodontology, University of Bari; and the patients gave written informed consent.

**Human osteoblasts**

Trabecular bone specimens, obtained from alveolar bone after informed consent from Pp patients and healthy donors in the Department of dentistry were cleaned of soft tissues, reduced to small fragments and digested with 0.5 mg/ml of Clostridium histolyticum neutral collagenase (Sigma Chemical Co, St Louis, Missouri) in phosphate-buffered saline (PBS) with gentle agitation for 30 min at 37°C (39-41).

Bone fragments were then washed (three times) with α-MEM (Gibco Ltd, Uxbridge, UK) containing 3.024 g/l sodium bicarbonate and cultured in medium supplemented with 10% FCS (Foetal Calf Serum; Gibco Ltd), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml amphotericin B and 50 IU/ml mycostatin, at 37°C in a water-saturated
TABLE I - PRIMERS FOR PCR ANALYSIS.

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<th>gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Ta</th>
<th>Numero di cicli</th>
<th>bp</th>
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<td>5'-gtagagttgatggctcagtg-3'</td>
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<td>25</td>
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<td>DcR2</td>
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<td>5'-ctccctcgctgctggtttt-3'</td>
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<td>25</td>
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<td>58</td>
<td>25</td>
<td>415</td>
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<tr>
<td>DR5</td>
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<td>5'-gtgatggatcatttcatgt-3'</td>
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Alkaline phosphatase assay

Alkaline Phosphatase (ALP) activity has been evaluated by histochemical and fluorimetric methods on human OBs obtained from Pp and Controls. Cells were cultured in α-MEM with 10% FCS and, once having reached the confluence of the cells, were stopped and used as Time 0 (T0) while the others were grown with the same medium supplemented with 50 µg/ml ascorbic acid and Dexametason 10⁻⁸ M for 5 (T5) and 10 (T10) days to obtain differentiation. Histochemical staining was performed with appropriate Sigma Kit used on semi-confluent Obs.

Biochemically, ALP activity was measured by a fluorimetric method, with 4-methyl-umbelliferyl-phosphate used as substrate. Monolayers were solubilized in 0.1% sodium dodecyl-sulphate (SDS). The cell lysates were incubated at pH 10.3 in the presence of 200 µmol/L substrate at 37°C for 30 min. The 4-methyl-umbelliferylone produced by the enzyme was detected by monitoring its fluorescence at 369-nm excitation and 448-nm emission wavelengths. The rate of production was converted into nmol/min from standard curves. Results were normalized per mg cell protein. Protein content was measured by BCA reagent kit (Bio-Rad, Hercules, CA).

Cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Obs were cultured in 96-well tissue-culture plates at a density of 3 x 10⁵ cells/well in α-MEM with 10% FCS. With different times of culture, 24 hours and 48 hours, TRAIL was added at a concentration of 0, 10, 50, 100 500 ng/ml. After 24 and 48 hours a volume of 200 µl/well of MTT (stock solution 5 mg/ml) at the final concentration of 0.5 mg/ml was added. The cultures were incubated for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. The reaction was stopped by the addition of 150 µl of acid cell lysis buffer (0.04 N HCl in absolute isopropanol). After mixing, the optical density was read at 570 nm using an automatic plate reader (550 Microplate Reader Bio-Rad Laboratories Inc., CA, USA). A reduction in the optical density was used as a measure of viability (apoptosis), normalized to cells incubated under control conditions.

Mineralized bone nodule formation

Human OBs were seeded in 6-well plates and cultured in α-MEM supplemented with 10% FCS, 50 µg/ml of ascorbic acid, 10mM of β-glycerophosphate, 10⁻⁸ M dexamethasone. The medium was changed every 3 days for 8 weeks and the mineralized matrix nodules were detected with Von Kossa staining. The cells were fixed with 3% para-formaldehyde for 10 minutes, stained with silver nitrate (AgNO₃), rinsed with distillated water, exposed for 1 hour to bright light and finally observed. The photomicrographs of mineral
nODULES FORMED IN CULTURES WERE OBTAINED USING A NIKON ECLIPSE E400 MICROSCOPE EQUIPPED WITH A NIKON PLAN FLUOR 10x/0.30 DICL. THE MICROSCOPE WAS CONNECTED WITH A NIKON DIGITAL CAMERA (DXM 1200); THE ACQUISITION AND THE QUANTITATIVE ANALYSIS OF THE MINERALIZED SURFACE WERE PERFORMED BY USING THE SOFTWARE LUCIA G VERSION 4.61 (BUILD 0.64) FOR NIKON ITALY. THE PERCENTAGE OF MINERAL NODULES WAS REPRESENTED BY THE HISTOGRAM.

RNA ISOLATION AND (RT)-PCR ANALYSIS

OBs OBTAINED FROM PP AND CONTROLS WERE CULTURED IN α-MEM WITH 10% FCS AND ONCE HAVING REACHED THE CONFLUENCE PART OF THE CELLS CULTURED WERE STOPPED AND USED AS TIME 0 (T0) WHILE OTHERS WERE GROWN WITH THE SAME MEDIUM SUPPLEMENTED WITH 50 µG/ML ASCORBIC ACID AND DEXAMETASONE 10^-8 M FOR 5 (T5) AND 10 (T10) DAYS TO OBTAIN DIFFERENTIATION. THEN OB WERE SUBJECTED TO mRNA EXTRACTION USING SPIN COLUMNS (RNEASY, QIAGEN, HILDEN, GERMANY) TO DETECT THE EXPRESSION OF DR4, DR5, DCr1, DCr2 AND THE HOUSEKEEPING GENES GLYCERALDEHYDE PHOSPHATE AND GAPDH SENSE: 5'-GGAGTCAACGGATTTGGT-3' DEHYDROGENASE (GAPDH). FIRST STRAND cDNA WAS GENERATED USING THE SUPERSCRPT FIRST-STRAND SYNTESIS SYSTEM KIT FOR.

FIG. 1A - HISTOCHEMICAL STAINING FOR ALP ON OSTEOCLASTS FROM PP VERSUS CONTROL AT DAYS 0, 5 AND 10.

FIG. 1B - BIOCHEMICAL ASSAY OF ALP ACTIVITY ON OSTEOCLASTS FROM PP VERSUS CONTROLS AT DAYS 0, 5 AND 10.

Alteration of activity and survival of osteoblasts

"Alteration of activity and survival of osteoblasts"
The cells were cultured to protein enzymatic levels.

The cells were obtained from patients and were biochemically used for further experiments.

The OBs Collagene was shown to be less efficient in the percentage of osteoblasts formed compared to the controls.

We obtained OBs from Pp that showed mineralization and comparison between the groups was performed by the t-test.

After both five and ten days of differentiation, the ALP before starting the differentiation process, from healthy donors. Control cells already express 10\(^{10}\) activity confirmed ALP while from cell (T10) obtained not both 5 differentiations.

The OBs obtained from Pp were cultured for 5 days and 10 days in a medium supplemented with 50 µg/ml ascorbic acid and Dexametasone 10\(^{-4}\)M of Pp and controls were harvested in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, and 1 mmol/L PMSF]. Proteins were determined by the BCA Protein assay Reagent Kit (Pierce Biotechnology, Rockford, MN USA). Fifty µg of cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK). Blotted filters were probed with: mouse anti-β actin (Chemicon International Inc.), DR4, DR5, DcR1,DcR2 polyclonal antibody (Peprotech) and Collagene (Santa Cruz, California-USA). After incubation with peroxidase-conjugated secondary antibodies, specific reactions were revealed with the ECL detection kit (Amersham Pharmacia).

**Western blot analysis**

OBs, after having reached the confluences, were cultured for 5 days and 10 days in a medium supplemented with 50 µg/ml ascorbic acid and Dexametasone 10\(^{-4}\)M of Pp and controls were harvested in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, and 1 mmol/L PMSF]. Proteins were determined by the BCA Protein assay Reagent Kit (Pierce Biotechnology, Rockford, MN USA). Fifty µg of cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK). Blotted filters were probed with: mouse anti-β actin (Chemicon International Inc.), DR4, DR5, DcR1,DcR2 polyclonal antibody (Peprotech) and Collagene I (Santa Cruz, California-USA). After incubation with peroxidase-conjugated secondary antibodies, specific reactions were revealed with the ECL detection kit (Amersham Pharmacia).

**ELISA assay**

Trail serum levels, in samples obtained from the Pp patients as well as from the controls were detected by commercially available enzyme-linked immunosorbent assay kits (ELISA, R&D Systems Inc.), according to the manufacturer’s instructions. The samples were diluted to the concentrations within the standard curve range. The absorption was determined with an ELISA reader (550 Microplate Reader, Bio-Rad Laboratories Inc., CA, USA) and the results were expressed as mean ± SE. The comparison between the groups was performed by the t-test.

**Statistical analyses**

Statistical analyses were performed by Student’s t-test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). The results were considered statistically significant for p<0.05.

**RESULTS**

**Osteoblasts obtained from Pp express lower levels of ALP**

The histochemical staining, performed on osteoblasts obtained from Pp to evidenced enzymatic level of ALP showed a weak stain significantly different with respect to the OBs obtained from healthy donors. Control cells already express the ALP before starting the differentiation process, while the staining is absent in the cells from the Pp. After both five and ten days of differentiation, the...
ALP staining was drastically higher in control OBs (Figure 1 A).

This result was further confirmed by biochemical assay. ALP activity was measured and quantified: OBs obtained from Pp express lower enzymatic levels. The result became evident after five days of differentiation (T5) and was remarkable after ten days (T10) (Fig. 1 B).

Osteoblasts obtained from Pp express lower levels of Collagene I

OBs from Pp do not increase the production of the collagen I protein during cell differentiation. The cells were cultured to obtain osteogenic differentiation for 0, 5 and 10 days. Collagene I levels were undetectable in both control and Pp cells in undifferentiated cells after 5 and 10 days of culture while OBs from control express Collagene I in the cells from Pp the protein was still undetectable (Fig. 2).

Osteoblasts obtained from Pp are less efficient in forming bone mineral matrix nodules

Long term cultures with the opportune medium allow osteoblast to form mineral matrix. Although osteoblasts from Pp formed mineral matrix nodules, the specific staining showed that the cells were less efficient than OB from controls. In the picture of Fig. 3 exemplificative samples from the experiment are shown. With the image analyzer we quantified the percentage of the area mineralized in fields where we cultured OB obtained from Pp in respect to the control. The percentage of mineralized surface is 38±3 in samples from Pp in respect to 85± 4 in the controls (Fig. 3).

Lower expression of DcR2 in OBs from Pp

We hypothesized a role played by Trail interfering with differentiation and surviving of OBs from Pp. Thus we studied the expression of TRAIL receptors at RNA and protein level by Western blot analysis and PCR. In Western blot analysis the death receptors DR4 and DR5 and the decoy receptor DcR1 are equally expressed by OBs from Pp and Controls (data not shown). Interestingly OBs from Pp express lower levels of DcR2 at the protein levels (Fig. 4 B); furthermore PCR analysis showed decreasing RNA levels according to cultures times (T0, T5 and T10) either in Pp OBs or in the controls, but all the values are lower in the OBs from Pp (Fig. 4 A). These data indicate that OBs from Pp should be more sensitive to the apoptotic effect of TRAIL being one of the decoy (DcR2) receptors less expressed either as protein, or as mRNA. The results show a role for TRAIL in decreasing OBs vitality and differentiating possibility in Patients affected by periodontal disease.

Fig. 4 - TRAIL levels in osteoblasts from Pp and controls at PCR (A) and Western Blot analyses (B).
already starting from a concentration of 10 ng/ml, while in OB from control the apoptotic effect started at 100 ng/ml (Fig. 5). After 48 hours the treatment with Trail determined apoptosis on both Pp and control osteoblasts but the effect was stronger in Pp, for example at the concentration of 500 ng/ml the percentage of apoptotic cells was 50% in OBs from control but reached 80% in OBs from Pp (Fig. 6).

TRAIL serum levels are higher in Pp

TRAIL serum level was measured in samples obtained from Pp and compared to samples from healthy donors. In Pp TRAIL concentration was 851±221 pg/ml (range 470-1236) while in Control patients the value was 389±247 pg/ml (range 100-806). This result emphasizes the in vitro data on the role of TRAIL in altered bone remodelling that occurs in Pp.

DISCUSSION

Periodontitis is a disease of bacterial aetiology and its progression may be related to both direct bacterial effects on host tissues and activation of a large complex of autocrine and paracrine factors, which can amplify the local inflammatory reactions with consequential periodontal and systemic damage (42-44). Alveolar bone loss is the weightiest event in periodontal disease; it certainly occurs consequently to an altered local bone remodelling. Thus bone cells, osteoblasts and osteoclasts, that are responsible for bone remodelling are involved in periodontal disease pathogenesis. Osteoclast
formation and differentiation are stimulated with the consequent event of an increased bone resorption. Increased bone resorption is determined by a local production of cytokine by T cells, B cells and osteoblasts (45-51). Also the OBs altered behaviour could modify bone remodelling reducing bone formation: this could be due to an increased cell apoptosis or to a decreased cell differentiation and activity, or to both.

We demonstrate, for the first time, that OBs obtained from alveolar bone of Pd patients are not well differentiated: in fact, they express a lower level of ALP; the lower activity of the enzyme has been measured by a biochemical assay. Furthermore, the OBs obtained from Pp are less efficient in producing bone mineral matrix nodules. All these three features indicate that OBs in Pp were involved in the unbalanced bone remodeling that developed locally during periodontal disease, driving the pathological process to alveolar bone resorption. At this point we wondered about the mechanism that in compromises osteoblast activity periodontal disease. We therefore hypothesized that in OBs from Pp apoptosis occurs in a faster and more efficient way. We investigated the apoptotic protein TRAIL and its death (DR4, DR5) and decoy (DcR 1, DcR2) receptors. We found a significant difference in DcR2 that is less expressed either as protein or RNA level in OBs from Pp. Thess data indicate that OBs from controls are more sensitive to the apoptotic effect of TRAIL being one of the decoy (DcR2) receptor less expressed. This result marks a role for TRAIL in decreasing OBs vitality in Patients affected by periodontal disease. This data is validated from the result that OB from Pp are more sensitive to the apoptotic effect of TRAIL and furthermore from the evidence that TRAIL serum levels are higher in patients affected by periodontal disease.

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The effect of porcine Orexin A on insulin plasma concentrations in pigs

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ABSTRACT: Orexin A is a member of a wider family of orexigenic neuropeptides that have been recently discovered. They are produced by a small group of neurons located in the area of the brain, round the nucleus of the fornix (posterior hypothalamus), in the paraventricular nucleus, the dorsomedial nucleus, the ventromedial hypothalamus, as well as in the lateral hypothalamic region; these are sites known to be involved in regulating feeding in mammals. Orexin A is a neuropeptide, which is involved in appetite regulation and energy homeostasis. An intracerebroventricular (i.c.v.) injection of Orexin A in the brain of rats causes an impressive increase in food consumption. In addition, a subcutaneous or intravenous (IV) injection of Orexin A produces changes on insulin plasma concentrations in rats. Recent research suggests that Orexin A is also involved in regulating many other physiological functions. In this study, we examined the potential effects of the central administration of porcine Orexin A on insulin plasma concentrations in pigs, and whether these changes are connected with the possible effect of the neuropeptide on the enteroinsular axis.

KEY WORDS: Orexin A, Insulin secretion, Gastrointestinal (or gut) hormones, i.c.v. injection, Enteroinsular axis, Brain-gut axis

Orexin A and Orexin B are two new neuropeptides discovered in 1998. They are produced by a small group of neurons located in the area of the brain, round the nucleus of the fornix (posterior hypothalamus), in the paraventricular nucleus, the dorsomedial nucleus, the ventromedial hypothalamus, as well as in the lateral hypothalamic region; these are sites known to be involved in regulating feeding in mammals (1, 2).

Despite the fact that the orexigenic neurons occupy a very small area of the CNS, their axes present an impressive dispersion to many different areas of the brain and the spinal cord. Thus, the large number of protrusions in the orexigenic neurons through their neuraxes suggests that the orexigenic circuit may possibly regulate a large number of physiological functions, including the one that takes part in the regulation of food intake (3-7). Both Orexins come from the same precursor peptide, prepro-Orexin, since the latter will undergo a certain series of proteolytic processes. The first 33 amino acids of the precursor peptide have the features of an important sequence, i.e., Orexin A with a molecular weight of 3562Da and a molecular structure identical to that found in humans, pigs, cattle, rats, and amphibians (8-10).

The action of Orexin A is directly related to the binding and activation of its corresponding receptor, which is denoted as OX1R (Orexin Receptor 1). This receptor is widespread in all species, and its activation is the result of intracellular Ca++ levels (1, 2, 8).

The brain-gut axis

Orexins (Orexin A, Orexin B) and their receptors are also present in the enteric nervous system and endocrine cells of the gut, suggesting that they may play a certain role both peripherally and centrally
in the regulation of feeding (11). Vagal and spinal primary afferent neurons, enteric neurons, and endocrine cells in both the gut and pancreas display Orexin and Orexin receptor-like immunoreactivity (12-15).

The enteroinsular axis
The connection between the gastrointestinal tract and the pancreatic islets has been described as the enteroinsular axis. It associates a network including nutrients, enteric hormones, and neural signals that all mediate or modulate the release of islet-cell hormones (16).

Published studies tried to confirm the orexigenic effect of Orexins when administered intracerebroventricularly (i.c.v.) in rats and compared with a strong stimulant of food, neuropeptide Y (NPY) and two weaker stimulants: a) (MCH) melanin-concentrating hormone and b) galanin (17-20). In addition, other studies examined the subcutaneous injection of Orexin A and its effect on insulin plasma concentrations in vivo and insulin secretion by perfused rat pancreas preparation in vitro (21-24).

Current research suggests that serum fasting insulin concentrations increase in a dose-dependent pattern, along with bolus subcutaneous administration of Orexin A in rats (21). Meanwhile, some changes in blood glucose concentration have been observed. Administration of Orexin A caused a statistically significant and dose-dependent increase in glucose concentration (21).

As far as we know, no study has yet been published on the effect of an i.c.v. administration of porcine Orexin A in fasting serum insulin with two different doses (5 and 10 mg), with or without simultaneous intravenous glucose injection in pigs. The duration of the experiment was 30 minutes, and all blood samples were collected 15 minutes prior to the beginning of each stage of the experiment, and immediately after the bolus, or in the beginning of continuous injection (0 minutes) and after 5, 15, and 30 minutes. The aim of this study is to investigate any possible connections between the effects of Orexin A on serum fasting concentration of insulin, and whether this experimental data can be correlated with the enteroinsular axis.

### Materials and Methods

#### Preparation of the experimental animals

The experimental protocol was applied in seven (12) pigs and was carried out at two different times, with a one-week interval.

The injection of the neuropeptide Orexin A (of porcine origin) was made into the lateral ventricle of the brain using a special catheter extracranially through a special valve placed at a previous time (1st time) before the injection (2nd time).

**Experimental protocol**

1st time

Placement of the special device (valve) in the skull of the experimental animal was done under general anesthesia. The point of cranial trepanation is determined at 1/3 of the distance (0.5 to 1cm from midline) between the midline at the boundaries of the frontal bone (frontal part) and the occipital bone and the zygomatic process of the frontal bone (Fig. 1).

2nd Time

The second time consists in the injection of the neuropeptide Orexin A into the lateral ventricle of the pig’s brain. This is achieved after puncture (under local anesthesia), using a lumbar puncture needle, 21G in diameter, and with the device implanted at the 1st time as guide, under general anesthesia.

The administration of porcine Orexin A was made as follows:

1) Rapid i.c.v. injection of the neuropeptide, in two doses of 5 and 10 mg, diluted in 0.5 ml of artificial cerebrospinal fluid (CSF), with a one-hour interval.

2) The afore-mentioned way of administration was repeated, this time with simultaneous intravenous glucose administration.

Insulin plasma levels were measured in samples of 3 ml for each measurement.

The collections of blood samples were made as follows:

1) 15 minutes prior to the injection;

2) Immediately after rapid injection, and also after 5, 15, and 30 minutes.

Following blood coagulation and centrifugation at 3000 rpm, the serum was collected in order to measure the levels of insulin. The serums were preserved at -70°C up to the date of the measurements. For the determination of insulin, a radioimmunoassay method with a standardized reagent of LINCO Research (1251 Radioimmunoassay Kit) was used.

**Statistical analysis**

The normal distribution of the sample’s variables was tested through the application of the Kolmogorov-Smirnov test (p>0.2). Several multivariate analyses of variance (MANOVAs) were
carried out, in order to investigate the changes in insulin plasma levels (µU/ml) after: a) rapid i.c.v. injection and b) rapid i.c.v. injection with simultaneous intravenous glucose administration, and with injection of: a) 0.5 ml of artificial CSF, b) 5 mg of porcine Orexin A, and c) 10 mg of porcine Orexin A.

Due to the existence of more than three pairwise comparisons (post hoc comparisons), Holm’s Sequential Bonferroni Adjustment was applied to each comparison. Through this adjustment, the probability of Type-I error occurring in the study was reduced, by calculating a different α for every comparison applied by pairs. The level of significance of the study, α, was set to 0.05.

The changes in the plasma levels of the above variables were investigated after: 1) rapid i.c.v. injection, 2) rapid i.c.v. injection with simultaneous intravenous glucose administration through injection of: a) 0.5 ml of artificial CSF, b) 5 mg of Orexin A at every time of the experiment (-15, 0, 5, 15, and 30 minutes) by applying repeated multivariate analyses of variance (repeated MANOVAs).

Insulin plasma levels are shown in the Tables and Figures as means±standard deviations. Processing of the sample data and drawing of figures were done through the application of SPSS 13.0 statistical software for Windows (2004, IL. USA).

Rapid intracerebroventricular (i.c.v.) injection of Orexin A (5 and 10 mg).

The artificial cerebrospinal fluid (aCSF) was administered in a dose of 0.5 ml, and no statistically significant changes in insulin plasma levels were seen during the study (p>0.05), as shown in Fig. 4.

RESULTS

Rapid i.c.v. injection of Orexin-A (5 and 10 µg)

The rapid i.c.v. injection of 5 mg of Orexin A resulted in a statistically significant increase in the value of plasma insulin throughout the experiment (p<0.001) (Fig. 4). The extra comparisons (post hoc pairwise comparisons) showed that the rapid i.c.v. injection of 5 mg of Orexin A caused a statistically significant increase in the value of insulin from (9.46±0.053 µU/ml) at time 0 to (14.27±0.076 µU/ml) 5 minutes later in the experiment (p<0.001). The value of insulin kept on increasing significantly and reached the values of (25.07±0.35 µU/ml) and (39.07±0.18 µU/ml) 15 and 30 minutes later in the experiment, respectively. Apart from the times of -15 and 0 minutes of the experiment, the insulin values at all other times are statistically different from one another (p<0.001) (Table I).

Similarly, the rapid i.c.v. injection of 10 mg of Orexin A caused a statistically significant increase in the value of plasma insulin during the experiment (p<0.001). Further comparisons, which are presented in detail on Table I, showed that there was a statistically significant increase in the value of insulin from (9.54±0.113 µU/ml) at time 0, to (27.23±0.221 µU/ml) 5 minutes later in the experiment (p<0.001).

In Fig. 4, we can see a statistically significant gradual increase in the value of plasma insulin following injections of 5 mg and 10 mg of Orexin A, at 30 out of 45 minutes of the experiment (p<0.001), which is not observed with the injection of the aCSF (p>0.05).

A statistically significant difference was found in the mean values of insulin between the rapid i.c.v. injections of the aCSF and Orexin A, 5 and 10 mg, at the different times of the experiment (p<0.001),
The effect of porcine Orexin A on insulin plasma concentrations in pigs

Fig. 2 - Mean insulin values following rapid i.c.v. injection of aCSF and Orexin A, in two doses of 5 and 10 mg in seven pigs, during the 45 minutes of the experiment.

Fig. 3 - Mean insulin values after rapid i.c.v. injection of aCSF and Orexin A, in two doses of 5 and 10 mg, with simultaneous intravenous glucose administration
as shown in Fig. 4.

After pairwise comparisons, statistically significant differences among the insulin values were found (Table II). This means that statistically significant differences were observed in plasma insulin levels during the rapid i.c.v. injection, at the time points of 5, 15 and 30 minutes of the experiment (p<0.001).

### Rapid (i.c.v.) injection of Orexin A (5 and 10 mg) with simultaneous intravenous glucose administration

During the rapid i.c.v. injection of 5 mg of Orexin A, a statistically significant increase was seen in the value of insulin from (9.40±0.58 µU/ml) in the beginning, to (30.41±0.318 µU/ml) at the first 5 minutes (p<0.001) and to (87.63±0.390 µU/ml) at the end of the experiment (p<0.001) (Fig. 5, Table III).

We see than with the simultaneous glucose administration, the increase in the value of insulin plasma is sharper (Fig. 5) compared with the previous values (Fig. 4).

Further comparisons showed statistically significant differences in the increase in insulin value starting from the first 5 minutes of the experiment (p<0.001), up to its completion (p<0.001) (Table IV).

### DISCUSSION

The presence of the neuropeptide Orexin A is due to the existence of the corresponding neurons, which are located in different parts of the brain and are in close vicinity to the corresponding neurons that produce and concentrate melatonin (25-26), since they present pairings, whereby they come into contact with glucose-sensitive neurons (27-29).

Both Orexin A and its corresponding receptor are also found outside the CNS. The multiple detections of Orexin A and its receptors, both in the CNS and peripherally, form a special neuronal orexigenic circuit/system (1-2, 8).

Despite the fact that the orexigenic neurons occupy a very small area in the CNS, their axes present an impressive dispersion in many different parts of the brain. The above anatomic finding also supports the involvement of Orexin A in many physiological functions and systems, such as the effect on food or water intake, energy homeostasis, the circadian timing system, the sleep-awakening and alertness cycle, the cardiovascular and endocrine systems, as well as on the sense of pain, thermogenesis, and temperature control of the organism (1-2-3, 7-8).

The aim of our study is to investigate the potential effects of the neuropeptide Orexin-A on insulin plasma secretion and release in pigs with or without simultaneous intravenous glucose injection. By recording any changes in insulin plasma levels, an attempt is made to understand the principles of function and determine the degree of participation/effect of Orexin A in the function of the enteroinsular axis, since the physiological actions of insulin implicate it directly or indirectly with the axis. In addition, there

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**TABLE I - CHANGES IN INSULIN PLASMA LEVELS (MU/ML) AT DIFFERENT POINTS IN TIME (MINUTES) AFTER RAPID I.C.V. INJECTION OF ACSF AND OREXIN A (5 AND 10MG).**

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Injection</th>
<th>aCSF(n=7)</th>
<th>Orexin-A,5mg(n=7)</th>
<th>Orexin A,10mg(n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15</td>
<td></td>
<td>9.44±0.151</td>
<td>9.43±0.076</td>
<td>9.23±0.16*</td>
</tr>
<tr>
<td>0</td>
<td>9.3 ±0.082</td>
<td></td>
<td>9.46±0.053</td>
<td>9.54±0.113</td>
</tr>
<tr>
<td>5</td>
<td>9.4±0.058</td>
<td></td>
<td>14.27±0.076</td>
<td>27.23±0.221</td>
</tr>
<tr>
<td>15</td>
<td>9.31±0.07</td>
<td></td>
<td>25.07±0.35</td>
<td>49.43±0.236</td>
</tr>
<tr>
<td>30</td>
<td>9.46±0.054</td>
<td></td>
<td>39.07±0.18</td>
<td>73.26±0.172</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation
are certain bibliographic references confirming the involvement of Orexin A, which acts directly on the axis and affects insulin plasma concentrations, by acting on the pancreatic islets (30-33).

The selection of insulin was made based on the criterion of its direct physiological correlation with the B cells of the pancreas. The latter are the place of origin of proinsulin which, prior to the secretion, is split into insulin and c-peptide, in equimolecular quantities (34-36). In addition, the selection of insulin was made after thinking that the pancreas is the main target-organ for the actions of Orexin-A neuropeptide (30).

The i.c.v. injection of Orexin A resembles conditions of lack of food in the lateral hypothalamus of pigs; as a result, the insulin plasma secretion rate is directly affected (17).

In the light of the above data, the changes in insulin levels before and after the (i.c.v.) injection of Orexin A were studied. Its administration caused an increase in the levels of the hormone in a time- and dose-dependent way (rapid injection). The simultaneous intravenous glucose administration led to an even larger increase in insulin plasma levels possibly due to the close vicinity of Orexin A neurons with those characterized as glucose-
TABLE III - CHANGES IN INSULIN PLASMA LEVELS (MU/ML) AT DIFFERENT POINTS IN TIME (MINUTES) AFTER RAPID I.C.V. INJECTION OF ACSF AND OREXIN A (5 AND 10MG) WITH SIMULTANEOUS INTRAVENOUS GLUCOSE ADMINISTRATION.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>aCSF(n=7)</th>
<th>Orexin A,5mg (n=7)</th>
<th>Orexin A,10mg(n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15</td>
<td>9.40±0.578</td>
<td>9.21±0.135</td>
<td>9.51±0.157*</td>
</tr>
<tr>
<td>0</td>
<td>9.54±0.113</td>
<td>9.40±0.58</td>
<td>9.54±0.162</td>
</tr>
<tr>
<td>5</td>
<td>9.54±0.013</td>
<td>30.41±0.318</td>
<td>40.17±0.189</td>
</tr>
<tr>
<td>15</td>
<td>9.51±0.157</td>
<td>55.69±0.204</td>
<td>68.37±0.256</td>
</tr>
<tr>
<td>30</td>
<td>9.53±0.534</td>
<td>87.63±0.390</td>
<td>94.16±0.122</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation

TABLE IV - PAIRWISE COMPARISONS OF INSULIN VALUES (MU/ML) AFTER RAPID I.C.V. INJECTION OF ACSF AND OREXIN A (5 AND 10MG) WITH SIMULTANEOUS INTRAVENOUS GLUCOSE ADMINISTRATION IN SEVEN PIGS.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Rapid i.c.v. Injection</th>
<th>P value of Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15</td>
<td>(1) aCSF</td>
<td>(1)-(2): p &gt; 0.05 SI</td>
</tr>
<tr>
<td></td>
<td>(2) Orexin A/5mg</td>
<td>(1)-(3): p &gt; 0.05 SI</td>
</tr>
<tr>
<td></td>
<td>(3) Orexin A/10mg</td>
<td>(2)-(3): p &gt; 0.05 SI</td>
</tr>
<tr>
<td>0</td>
<td>(1) aCSF</td>
<td>(1)-(2): p &gt; 0.05 SI</td>
</tr>
<tr>
<td></td>
<td>(2) Orexin A/5mg</td>
<td>(1)-(3): p &gt; 0.05 SI</td>
</tr>
<tr>
<td></td>
<td>(3) Orexin A/10mg</td>
<td>(2)-(3): p &gt; 0.05 SI</td>
</tr>
<tr>
<td>5</td>
<td>(1) aCSF</td>
<td>(1)-(2): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(2) Orexin A/5mg</td>
<td>(1)-(3): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(3) Orexin A/10mg</td>
<td>(2)-(3): p &lt; 0.001 *</td>
</tr>
<tr>
<td>15</td>
<td>(1) aCSF</td>
<td>(1)-(2): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(2) Orexin A/5mg</td>
<td>(1)-(3): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(3) Orexin A/10mg</td>
<td>(2)-(3): p &lt; 0.001 *</td>
</tr>
<tr>
<td>30</td>
<td>(1) aCSF</td>
<td>(1)-(2): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(2) Orexin A/5mg</td>
<td>(1)-(3): p &lt; 0.001 *</td>
</tr>
<tr>
<td></td>
<td>(3) Orexin A/10mg</td>
<td>(2)-(3): p &lt; 0.001 *</td>
</tr>
</tbody>
</table>

SI: Statistically Insignificant
*Statistically significant
The effect of porcine Orexin A on insulin plasma concentrations in pigs

sensitive, where the latter are mainly excited by the presence of glucose, but also by the doses of 5 and 10 mg of the neuropeptide.

Other researchers studied the release of Orexin A in experimental conditions in vitro (i.e. in isolated pancreatic islets of rats) compared with extracellular glucose concentrations, and found that there is an increase in glucose concentrations.

The same samples were used in order to measure insulin concentrations, which increase. In addition, the same researchers administered Orexin A subcutaneously in rats and found a reduction in insulin plasma levels (37). However, there is a bibliographic reference arguing that the intravenous administration of Orexin A finally produces an increase in insulin plasma levels in rats (21).

In another experimental pattern provided by other authors, using however, the same experimental animal (rat), the continuous intravenous injection of Orexin A was applied for a time period of 30 minutes, in ever-increasing injection doses. These were followed by successive collections of blood samples at times 0, 10, 20, and 30 minutes after injection, and it was found that there was no statistically significant change in insulin plasma concentrations (30).

The above different biological behaviors of insulin, as described in this study, may be due to the different experimental parameters used by researchers (i.e., different experimental animal and type of insulin stimulation, different quantity and origin of Orexin A, as well as different time periods chosen for taking blood samples).

There are no experimental studies on pigs, or other experimental animals, in the international literature correlating an i.c.v. injection of porcine Orexin A in these doses of 5 and 10 mg, in rapid way of administration, with the simultaneous administration of glucose and the subsequent collection of blood samples at 5 successive times for the determination of insulin plasma levels.

In conclusion, an i.c.v. injection of porcine Orexin A into the lateral ventricle of the pig’s brain causes increases in plasma insulin concentrations, in a time- and dose-dependent way. When the i.c.v. injection of porcine Orexin A is accompanied by the simultaneous intravenous glucose injection, the levels of these increases in insulin plasma concentrations are even larger.

These findings indicate the potential regulatory role of Orexin A in the function of the enteroinsular axis.

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