Roum. Biotechnol. Lett., Vol. 7, No. 6, 2002, pp 995-1002

ORIGINAL PAPERS

Matrix Metalloproteases Involved in Injured Corneal Tissue Remodelling

LUCIA MOLDOVAN[*], ANISOARA CAMPEAN[**], OTILIA ZARNESCU[**], MARINELA FLAMANZEANU[*], CLAUDIA NICOLAE[*], DANA DUMITRESCU[***]

[*]*National Institute for Biological Sciences, 296 Splaiul Independentei, 77748 Bucharest 6, P.O.Box 17-16, Romania, e-mail: luci@ibd.dbio.ro*
[**]*Faculty of Biology, University of Bucharest, 91-95 Splaiul Independentei, 76201 Bucharest 5, Romania*
[***]*Municipal Hospital, Ploiesti, Romania*

Received: 7th July 2002; Accepted: 14th August 2002

Abstract

Our aim was to demonstrate the matrix metalloproteases role in corneal remodelling after injury by keratotomy and chemical burn and to study their modulation by dexamethasone.

The study was performed on 3 groups of albinotic rabbits which were injured as follows: a) mechanical injury 0.1 mm depth in cornea; b) chemical burn by NaOH 1N; c) chemical burn followed by dexamethasone treatment. After sacrification, rabbit corneas were analysed by light and electron microscopy and gelatin-zymography.

In normal cornea the constant presence of pro-enzymatic form of MMP-2 was observed. After mechanical injury, MMP-2 level rises and the active form of MMP-2 becomes present as well. Moreover, the temporary presence of gelatinase B (MMP-9) was observed, with a peak at 10 days after injury. In the case of cornea injury by chemical burn, we found an overexpression of MMP-9 compared to the mechanically injured cornea and the presence of this enzyme at 30 days after injury. Increased proteolytic activity of gelatinase B is correlated with incomplete renewal of the epithelial basement membrane. Following dexamethasone treatment of injured cornea, gradual repression of both latent and active forms of MMP-9 was observed. After 7 days of treatment, total repression of active MMP-9 occurs and after 14 days the gelatinolytic activity of the zymogen form dissapears as well.

In conclusion, MMP-2 has a functional role in normal cornea, catalyzing degradation of occasionally altered collagen molecules. After injury, this enzyme participates in a long term remodelling of collagen in the corneal stroma, for the functional renewal of the tissue. MMP-9 has an important role in re-epithelialization control and formation of the epithelial basement membrane.

Keywords: cornea, matrix metalloproteases, remodelling, chemical burns, mechanical injury, gelatinase

Introduction

Cornea is composed of three distinct layers of cells associated with their extracellular matrix (ECM). The structure of the corneal tissue is controlled by the dynamic, interactive correlation between cells and the ECM that they secrete. During embryonic development or metamorphosis, remodelling of the ECM by cells is an important process that induces pattern formation. The adult tissue is structurally more static, but in the injured status, the homeostatic demands may induce a dramatic return to rapid remodelling (Davison and Galavy, 1986). In ECM remodelling, very important is the specialized mechanism that mediates the turnover of its macromolecules, such as collagen and proteoglycans (Matsubara et al., 1991; Fini et al., 1992).

In the case of cornea injury, the structural and supramolecular organization of matrix components becomes unbalanced and this eventually leads to loss of sight (Fini et al., 1990). The molecular basis of degradation of ECM components is dependent on the action of proteolytic enzymes known as matrix metalloproteases (MMPs) (Sotozno et al., 1999). There is a balance between ECM degradation by MMPs and the inhibition of their activity by tissue and endogenous proteic inhibitors. Any modification of this balance can lead to fibrotic processes (when the concentration of the inhibitor is bigger than that of the enzyme) or to excessive degradation of ECM (when the concentration of the inhibitor is smaller than that of the enzyme).

MMPs, also named matrixins, represent a family of zinc endopeptidases structurally and functionally related, capable of degrading all the proteic components of ECM (collagens, proteoglycans, laminin, fibronectin etc.) both *in vitro* and *in vivo* (Nagase et al., 1992; Fini et al., 1996). Excepting MMPs from neutrophils (collagenase 2 and gelatinase B), which are deposited in secondary and tertiary granules, from where they can be rapidly released in response to specific stimuli, the synthesis and activity of MMPs are highly regulated. Normal tissues do not deposit MMPs and their constitutive expression is low (Matrisian, 1992). Because MMPs have the capacity to catalyze degradation of structural components of ECM, it was proposed that their main function is physiological tissue remodelling during embryonic development, uterine cycle, post-partum uterine involution and wound healing. Aberrant expression of MMPs may determine tissue modifications, being associated with pathological processes such as ostheoarthritis, rheumatoid arthritis, atherosclerothic plaque rupture, aorthic anevrism and tumor progression (Shapiro et al., 1999).

The aim of this paper is the evidentiation of MMPs and of their role in normal cornea and in corneal remodelling after injury by keratotomy and chemical burn.

Materials and Methods

Animals

We used albinotic rabbits aged 4-6 months, and general anaesthesis was performed by subcutaneous administration of sodium phenobarbital. Corneas were as well anaesthetized by dropping xylin. Animals were divided in several groups. We performed a 0.1 mm depth incision in the cornea of rabbits in the first group and a chemical burn with 1N NaOH in the cornea of animals from the second group. Immediately after injury, all the eyes of the animals were treated with gentamycin ophtalmic solution to avoid infection. Under anaesthesis, the animals were killed. Samples as large as 2mm in diameter were taken from normal and injured cornea, after 3 time points: 3 days, 10 days and 30 days. Another group of chemically burned cornea was treated with ophtalmic solution containing dexamethasone 0.5 mg/ml for

14 days. The animals were sacrificied and corneas were analysed by light microscopy (LM), electron microscopy (EM) and gelatin-zymography.

Tissue preparation

For light microscopy, the corneal samples were fixed in Bouin's fixative, embedded in paraffine, sectioned at 7 μm and stained with Azan. For electron microscopy, the corneal specimens were immersed in a fixative containing 2.5% glutaraldehide and 2% paraformaldehide in 0.1M cacodylate buffer (pH 7.4), cut into small pieces of about 1x2 mm and postfixed with 1% OsO4 in 0.1M cacodylate buffer at 40^0C for 2 hours. They were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut with an LKB ultratome, stained with uranyl acetate and lead citrate and examined in transmission electron microscopy (Philips).

MMPs isolation

MMPs isolation from corneal tissues was performed using 2M GuHCl in 0.05 M Tris-HCl buffer pH 7.4. After several centrifugations, the obtained solution was dialysed against the same buffer.

Protein assay

Protein concentration of corneal tissues was determined using the Amido Black protein staining method elaborated by Sheffield (1987). The value range was 1.45 – 1.90 μg/ml.

MMPs assay

MMP presence in corneal tissues was assessed using the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gelatin zymography (Cimpean et al., 1998). Using this technique, both active and latent species can be visualised. Type I gelatin was added to standard Laemmli acrylamide polymerization mixture to a final concentration of 1mg/ml. Samples were then mixed 3:1 with substrate gel sample buffer (10%SDS, 4% sucrose, 0.25M Tris-HCl pH 6.8 and 0.1% bromphenol blue) and loaded onto the gel without boiling at a protein concentration of 15 μg. After electrophoresis, gels were washed in 50 mM Tris-HCl, 5mM $CaCl_2$, 1mM $ZnCl_2$, pH 7.6, containing 0.5% Triton-X100 for 1 hour, and incubated in the same buffer with 1% Triton-X100 for 18 hours. After incubation, gels were stained for 1 hour in 0.1% Coomasie Brilliant Blue R-250 in 30% methanol and 7% acetic acid and destained in 30% methanol and 10% acetic acid. Gelatinolytic (MMP-2 and MMP-9) activity was visualised as zones of clearance within the gels.

Results and Discussion

In our studies performed on tissue extracts obtained from normal and injured cornea, in order to evidence the presence of gelatinolytic activity characteristic to some MMPs, we demonstrated the variable presence of several members of the MMPs family.

In normal cornea, gelatin zymography (**Figure 1** – lane a) showed the constant presence of pro-MMP-2 (approximative 70 kDa) and of homodimers MMP-2 (approximative 120 kDa), suggesting that this is a normal constituent of cornea, which plays a role in degradation of occasionally altered collagen molecules.

After injury by keratotomy, MMP-2 is present both as pro-enzymatic and active form (approximative 62 kDa) (**Figure 2** – lanes b, c, d), this beeing correlated with epithelial cells migration into the wounded area and with increased turnover of collagens in the repair

process. In this case it can be also noticed the temporary presence of gelatinase B (MMP-9) in zymogen form (approximative 96 kDa) but also as partially activated form (approximative 88 kDa) with a peak at 10 days after injury, thus demonstrating that gelatinase B is not involved in long term corneal remodelling, but in re-epithelialization.

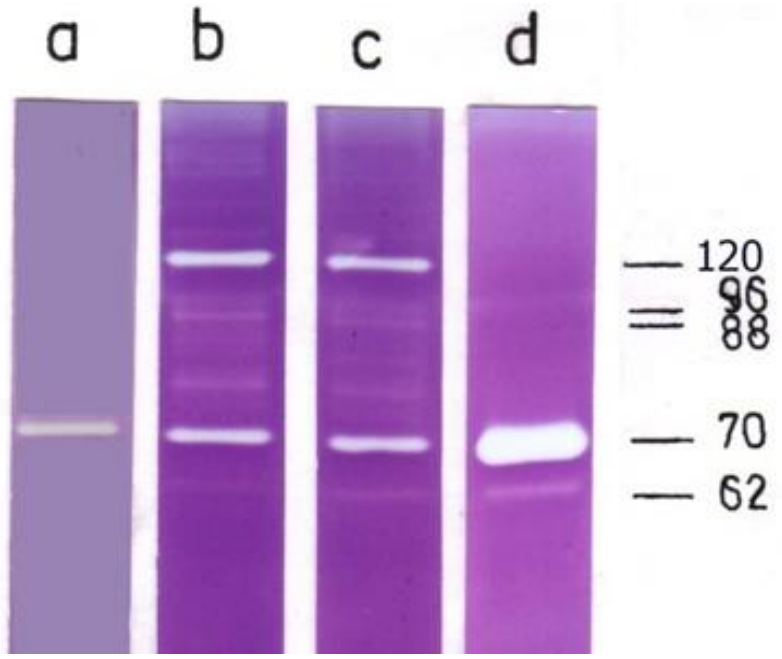

Figure 1 – Characterization of gelatinolytic activities present in normal cornea (a) and mechanically wounded cornea at 3 days (b), 10 days (c) and 30 days (d).

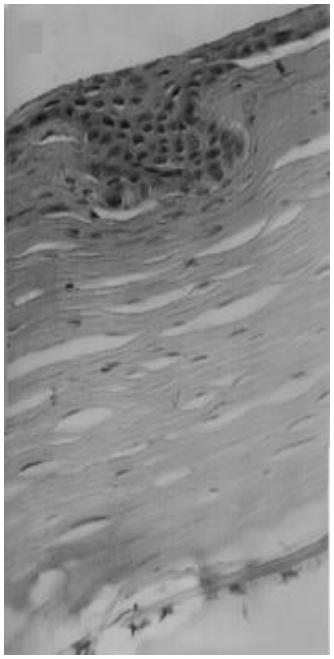

Figura 2 - Light microscopy image of mechanically injured rabbit cornea 3 days after keratotomy. Hematoxylin-eosin staining was performed.

Gelatinase B or MMP-9, in active form, present in injured tissue was differently expressed compared to the other MMPs. Thus, the level of this enzyme did not gradually rise within the 3-10 days period but after 30 days it was not detectable anymore (**Figure 1**). This can be explained by the gradual reduction and final disappearence of the inflammatory process resulted after cornea injury. Unlike MMP-2, which is constitutively expressed by most of the cells, MMP-9, initially identified as an enzyme secreted by polymorphonuclear cells and activated macrophages, can be induced by different stimuli in different cell types. This is how it can be explained the presence of this enzyme both in latent and active forms in the injured cornea.

It was observed as well that the expression of gelatinase B is displayed in basement cells of the epithelium and migrates until close to the corneal wound. These data show that MMP-9, as an active form, is not involved in stromal remodelling, which occurs on a long time scale, but plays an important role in epithelial renewal. Moreover, it is known that gelatinases can degrade components of epithelial basement membranes. It was observed that loss of gelatinase coincides with the formation of collagen VII anchor fibrils (substrate of this enzyme), in the area of the epithelial basement membrane (Fini et al., 1998).

Light microscopy showed that these modifications coincide with migration of stromal cells in the wounded area and with intiation of cell division and repaired matrix deposition (**Figure 2**).

The study of the kinetics of both MMPs' expression during deposition of renewed tissue showed that they reached a peak 10 days after injury and after that decreased heavily. Thus, at 30 days (**Figure** 1 - lane d) only pro-MMP-2 and MMP-2 were present. This suggests that 30 days after injury a normal physiological state of tissue remodelling is reached.

Tissue interactions play an important role in induction and modulation of MMPs expression in early healed stromal tissue. In skin wounds, platelets that form the blood clot release increased quantities of growth factors and other cytokines that induce collagenase expression. A fibrin clot can be formed in cornea wounds as well, but it lacks blood elements. However, macrophages and other inflammatory cells migrate into cornea soon after injury occurs and they secrete, as the platelets, molecules that induce expression of MMPs, such as collagenase (Fini et al., 1998).

Corneal epithelial cells secrete as well substances that control collagenase expression. Recently it was identified that the principal inducer is interleukin IL-1α (Reim et al., 1997) and the principal repressor is the transforming growth factor TGF-β2. Corneal epithelial cells were shown to release IL-1α more efficiently in cell culture, that simulates the edge of a healing wound (Boisjoly et al., 1993).

Cell culture studies showed that these interactions result in modifications of the cellular phenotype and in secretion of the autocrine cytokine IL-1α, which controls collagenase expression. The synthesis of this cytokinin occured not only in injured cornea but as well in normal cornea. IL-1α controls both collagenase expression and expression of other cytokinins that are important in inflammation and wound healing, such as interleukin IL-8 (Kinoshita et al., 2001).

After injury by chemical burn, the MMP pattern is almost similar to that corresponding to mechanical burn. The only difference is that the intensity of the gelatinolytic band corresponding to MMP-9 is stronger in this case (**Figure 3**). This suggests an overexpression of this enzyme after chemical burn. Moreover, the presence of MMP-9 was observed as well at 30 days after injury, but of a lower intensity.

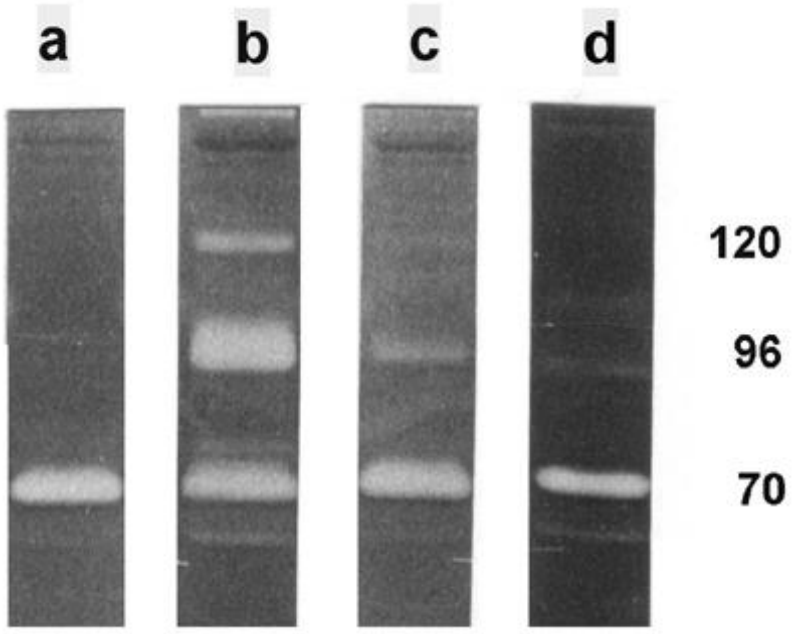

Figure 3 – Characterization of gelatinolytic activities present in normal cornea (a) and chemically wounded cornea at 3 days (b), 10 days (c) and 30 days (d).

Zymographic studies were compared with electron microscopy studies of epithelium and epithelial basement membrane renewal, in both mechanical and chemical injuries. 30 days after mechanical injury, both the epithelium and the basement membrane were completely renewed (**Figure 4**). In contrast, in the case of chemical injury only a partially synthesized basement membrane is observed (**Figure 5**).

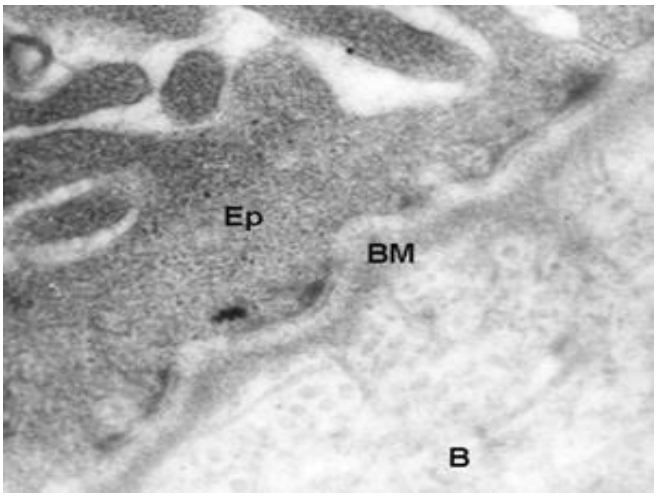

Figure 4 - Electron microscopy image of basement epithelial membrane at 30 days after mechanical injury of rabbit cornea. Ep-epithelium, BM- basement membrane, B-Bowman layer

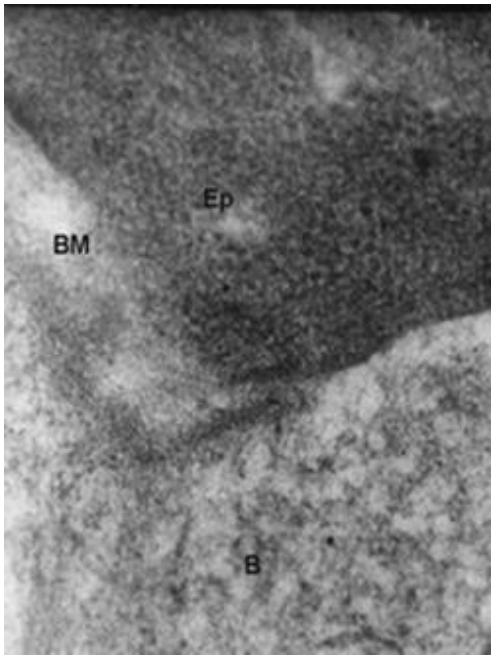

Figure 5 - Electron microscopy image of basement epithelial membrane at 30 days after chemical burn of rabbit cornea. Ep-epithelium, BM- basement membrane, B-Bowman layer

These results show that incomplete renewal of basement membrane could be due to excessive proteolytic activity of gelatinase B, which was present in high amounts in chemically burned cornea. The enzyme appears at 3 days as well as at 30 days after injury, thus demonstrating that MMP-9 is not a simple product of inflammatory cells that accumulate in the cornea after burn, but that it is synthesized by corneal cells themselves.

After dexamethasone treatment of chemically burned cornea, a gradual inhibition of latent and active forms of MMP-9 was noticed. Thus, 1 day after treatment both forms of MMP-9 are weakly present, pro MMP-9 (96 kDa) being more prominent. 7 days after treatment, total inhibition of MMP-9 (88 kDa) is found and at 14 days the gelatinolytic activity corresponding to the zymogen form (96 kDa) disapears as well (**Figure 6**).

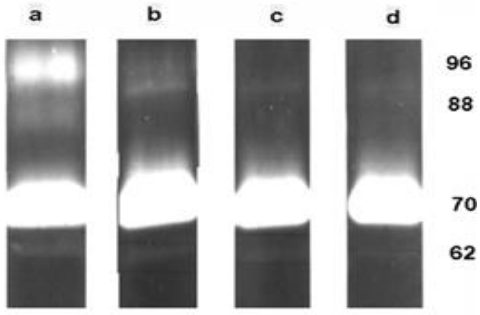

Figure 6 - Modulation by dexamethasone of MMP expression in vivo in injured rabbit cornea a-chemically burned cornea b, c, d-chemically burned corneas treated with dexamethasone for 1, 7 and 14 days respectively.

Glucocorticoids are known to be strong immunosuppressive agents and were used for a long time for treatment of inflammatory diseases including those mediated by T cells. They suppress some immunologic activities of monocytes and macrophages: cytokines and prostaglandins secretion, expression of cell surface receptors for complement and immunoglobins, fagocytosis and pinocytosis. They inhibit as well other functions of T cells like secretion of IL-2 and mitogenic ability.

Previous studies showed that glucocorticoids like dexamethasone and hydrocortisol inhibit synthesis of MMPs by fibroblasts and chondrocytes at transcriptional level, dependent on elements in the gene promotor (McGuire-Goldring et al., 1983; Brinckerhoff and Harris, 1981; DiBattista et al., 1991)). The half-life of MMP mRNA is not affected by glucocorticoids.

Our studies on chemically burned cornea proved that dexamethasone gradually inhibits MMP-9 expression in corneal cells.

Conclusions

- MMP-2 has a functional role in normal cornea, catalyzing degradation of occasionally altered collagen molecules. After injury by keratotomy or chemical burn, this enzyme participates in a long term remodelling of the corneal extracellular matrix, for the functional renewal of the tissue.
- MMP-9 has an important role in re-epithelialization control and formation of the epithelial basement membrane.
- Dexamethasone treatment showed that this glucocorticoid inhibits MMP-9 synthesis by corneal cells. Our results prove that dexamethasone is a potential therapeutic drug for MMP inhibition.

References

1. P.F. Davison, E.J. Galbavy - *Invest. Ophthalmol Visual Sci.*, **27**, 1478-1484 (1986).
2. M. Matsubara, M.T. Girard, C.L. Kublin, C. Cintron, M.E. Fini – *Dev. Biol.*, **147**, 425-439 (1991).
3. M. E. Fini, M.T. Girard, M. Matsubara – *Acta Ophthalmol.*, **70(Suppl.202)**, (1992).
4. M. E. Fini, M.T. Girard – *Invest. Ophthalmol Visual Sci.*, **31**, 1779-1788 (1990).
5. C.Sotozono, J. He, M. Tei, Y. Honma, S. Kinoshita - *Invest. Ophthalmol Visual Sci.*, **40**, 2430-2434 (1999).
6. H. Nagase, A.J. Barrett, J.F. Woessner, Jr. – *Matrix* **Suppl.1**, 421-424 (1992).
7. M. E. Fini, W.C. Parks, W.B. Rinehart, M.T. Girard, M. Matsubara, J.R. Cook, J.A. West-Mays, P.M. Sadow, R.E. Burgeson, J.J. Jeffrey, M.B. Raizman, R.R. Krueger, J.D. Zieske – *Am. J. Pathol.*, **149(4)**, 1287-1302 (1996).
8. L.M. Matrisian – *Bioessays*, **14**, 455-463 (1992).
9. S.D. Shapiro, R.M. Senior – *Am. J. Respir. Cell Mol. Biol.*, **20**, 1100-1102 (1999).
10. A. Cimpean, M. Caloianu, O. Zarnescu, L. Moldovan, N. Efimov, O. Ranga – *J. Med. Biochem.*, **2(4)**, 313-322 (1998).
11. G. Sheffield – *Anal. Biochem.*, **166**, 49-54 (1987).
12. M. E. Fini, J.R. Cook, R. Mohan – *Arch. Dermatol. Res.*, **Suppl. 290**, S12-S23 (1998).
13. M.Reim, A.Kottek, N.Schrage – *Prog. Ret. Eye Res.*, **16(2)**, 183-225 (1997).
14. H.M. Boisjoly, C.Laplante, S.F. Bernatchez, C.Salesse, M.Giasson, M.C.Joly – *Exp. Eye Res*, **57**, 293-300 (1993).
15. S. Kinoshita, W. Adachi, C. Sotozono, K. Nishida, N. Yokoi, A.J.Quantock, K.Okubo - *Prog. Ret. Eye Res.*, **20 (5)**, 639-673 (2001).
16. M.K.B. McGuire-Goldring, G. Murphy, M. Gowen, J.E. Meats, N.M. Ebsworth, C. Poll, J.J. Reynolds, R.G.G. Russel – *Biochim. Biophys. Acta*, **763**, 129-139 (1983).
17. C.E. Brinckerhoff, E.D.J. Harris - *Biochim. Biophys. Acta*, **677**, 424-432 (1981).
18. J.A. DiBattista, J. Martell-Pelletier, L.O.Wosu, T. Sandor, T. Antakly, J.P. Pelletier – *J. Clin. Endocrinol. Metab.*, **72**, 316-326 (1991).