MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES IN VARICOSE VEINS

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Abstract: Recent studies indicate the association of matrix metalloproteinases (MMPs) transformation from varicose veins with tissue inhibitors of metalloproteinases (TIMPs) activity alterations. This study aims to investigate MMPs and TIMPs in varicose veins, corresponding to CEAP II-III stages (simple varices) and CEAP IV-VI (complicated varices), following markers distribution profile, their morphology being associated to the clincal stage. The group of study was composed of 13 patients with varicose disease: 8 cases in CEAP II-III stage, 5 cases in IV-VI CEAP stage. After surgical treatment, the prelevated fragments, fixed in buffered formalin, were paraffin-embedded and initially processed for histopathological examination. In order to perform immunohistochemical analysis, anti-MMP-2, anti-MMP-9, anti-TIMP-1 antibodies were used. For both investigated groups, our results revealed evident differences between the two investigated MMPs. In a small number of analyzed cases we evidentiated an inconstant MMP-2 presence. On the other hand, MMP-9 was expressed in all cases, in a parallel manner to TIMP-1. Consequently, our data indicate a MMP-9 and TIMP-1 involvement in venous insuffiency matrix remodeling.

INTRODUCTION

Chronic venous insufficiency is a chronic inflammatory disease produced by the persistent and continuous effect of venous hypertension, with direct impact on the morphological structure of the venous wall. The role of valvular insuffiency, as a primary etiopathogenic factor of venous hypertension onset is currently contested (Rose, Ahmed, 1986, Travers et al., 1996, Gillespie et al., 2002), considering that etiologic factors responsible for the appearance of structural alterations are incompletely defined (Woodside et al., 2003). In venous wall remodeling several cells are involved: macrophages, smooth muscular cells, endothelial cells and fibroblasts (Woodside et al., 2003). Despite this, pathogenic phenomena sequence connecting the hemodinamics transformations specific for the venous hypertension and structural alterations of the venous wall represents a subject with many unknown issues. Several studies of tissular, cellular, and molecular modifications of the venous wall resulted in the modern etiopathogenic theory of the varicose vein that stresses on the leukocyte activation mechanism, the fibroblasts function alteration and the qualitative and quantitative matrix transformation.

The quantitative decrease of desmosine, isodesmosine and of the elastine: collagen rate in varicose veins, comparative with normal veins (Venturi et al., 1996), and also the fact that leukocyte activation, induced by hypoxia, is followed by free radicals liberation and proteases activation that results in extracellular matrix degradation (Michiels et al., 1997) have been demonstrated. On the other hand, hypoxia activates the endothelial cells that synthesize growth factors and stimulates the extracellular matrix production and deposition. This certifies that a major imbalance between structural components synthesis and degradation appears in varicose veins resulting in the loss of venous tonus, followed by dilatation and venous elongation.

In matrix proteins synthesis and degradation, MMPs and TIMPs have an important role (Travers et al., 1996, Venturi et al., 1996, Kockx et al., 1997, Michiels et al., 1997, Verbeuren et al., 1998, Badier-Commander et al., 2000, Gillespie et al., 2002). Recent studies indicate the association of matrix metalloproteinases transformation from varicose veins with MMPs activity alterations (Hanemaaijer et al., 1993, Vaalmo et al., 1997, Li et al., 2000). MMPs and TIMPs are temporarily produced as a response to external signals as: different proteases, cytokines or growth factors, cell-matrix, and cell-to-cell interactions. The review of the literature reveals a MMP-2 decrease and a TIMP-1 growth in varicose veins, comparative to normal veins (Porto et al., 1995), MMP-1 and MMP-13 low levels in veins located in the inferior part of the body, comparative with the veins located in the superior part of the body (Gillespie et al., 2002), contradictory findings regarding TIMP-1 and TIMP-2 presence and level (Johnson et al., 2001) as well as differences of the degree of expression and MMPs and TIMPs location in different venous territories (Woodside et al., 2003).

According to literature data, MMPs intervention in matrix components degradation (collagen, elastine, fibronectin, laminin), components that are synthesized by smooth muscular cells and by fibroblasts, is extremely variable (Borden, Heller, 1997), so a pattern is not yet established. In this context, this study aims to investigate MMPs and TIMPs in varicose veins, corresponding to CEAP II-III stages (simple varices) and CEAP IV-VI (complicated varices), following markers distribution profile, related to the morphology associated to the clincal stage.

MATERIALS AND METHOD

The group of study was composed of 13 patients with varicose disease: 8 cases in CEAP II-III stage, 5 cases in IV-VI CEAP stage, hospitalized in the "Dr. Victor Babes" Diagnosis and Treatment Center, Bucharest. Clinically, the patients in CEAP II-III stage presented varicose dilatations in the inferior limbs, associated with edema, and the patients in CEAP IV-VI had, as supplementary manifestations, cutaneous trophic alterations (hyperpigmentation- 5 cases, white atrophy as healed ulcer significance- 1 case, and active ulcer – 2 cases) and varicophlebitis (2 cases, 1 case with the involvement of the internal saphene on the entire lengtha and 1 case with the involvement of the leg varicose package).

All cases were surgically treated, by large crossectomy of the internal saphene, segmentary saphenectomies and perforans veins ligature, after pre-operatory echographic marking. From each patient, 1-2 cm fragments from internal saphene cross, internal saphene vein, leg varicose package and perforans veins were prelevated from the surgical specimen.

Prelevated fragments, fixed in buffered formalin, were paraffin-embedded and initially processed for histopathological examination. In order to perform immunohistochemical analysis, anti-MMP-2 antibodies (Thermo, A-GErVC2 clone, 1:250 dilution), anti-MMP-9 antibodies (Novocastra, policlonal, 1:100 dilution), anti-TIMP-1 (Novocastra, clone 6F6a, 1:100 dilution) and ABC kit (Vector, Burlingame), according to indirect tristadial Avidin-Biotin-Peroxidase (ABC), were used (Hsu, 1981).

3 μm thickness sections were displayed on Poly-l-Lysine coated slides, deparaffinized, hydrated, and treated with 3% hydrogen peroxide, for 30 minutes, to quench endogenous peroxidase. After washing in phosphate buffered saline (PBS) pH 7, non-specific binding sites blocking was performed by adding normal serum for 20 minutes. Overnight incubation with specific dilutions of the primary antibodies was performed in a wet chamber, at room temperature.

Secondary antibody was applied after 20 minutes washing in PBS, and sections were incubated 30 minutes, at room temperature, followed by 45 minutes incubation with ABC complex. After tap water washing, the developing was achieved in diaminobenzidine (DAB) solution (10 mg DAB in 88 ml PBS) with 0.0025 ml hydrogen peroxide. In the end, sections were counterstained with Meyer hematoxiline, dehydrated, clarified and mounted in an inorganic synthetic medium.

MMP-2, MMP-9 and TIMP-1 expressions were semi-quantitatively appreciated, according to the following grading scale: + for low intensity, ++ for moderate intensity, and +++ for strong intensity.

RESULTS

Histopathological exam revealed:

MMP-2, MMP-9, TIMP-1 expression in simple varices – CEAP II-III stage

MMP-2 reactions were negative in 6 of the 8 investigated cases, in all the prelevated fragments. Low intensity positivity was present in only two cases, in a discontinuous manner in endothelium and dispersed, in adventicial *vasa vasorum*. MMP-9 and its specific inhibitor, TIMP-1, had a moderate to strong reaction in all 8 cases. Regarding the localization, reactions were identified in endothelium and in the subendothelial tissue, around muscular cells from the medial and adventiceal layers, in *vasa vasorum* (fig. 1) and in the adjacent connective tissue cells. No significant differences in intensity and location between cross, internal saphene vein, leg varicose package, and perforans veins were noted.

MMP-2, MMP-9, TIMP-1 expression in simple varices – CEAP III-IV stage

MMP-2 reactions were negative in 3 of 5 investigated cases, in all prelevated fragments. Low and moderate intensity was noted in the other two cases, in neoformation capillaries developed inside the intima and in the medial connective tissue, and in the adventiceal *vasa vasorum* (fig. 2).

MMP-9 and its specific inhibitor, TIMP-1 reactions were present in the 5 cases, the intensity being variable. MMP-9 was positive in endothelium (fig.3), discontinuous, the color intensity being low, as well as in intramural and adventiceal neoformation capillaries, with a medium intensity staining. TIMP-1 was absent in endothelium and present, with high intensity, in intramural neoformation capillaries, *vasa vasorum* and adventiceal cells (fig. 4). No major

differences of intensity and location between cross, internal saphene vein, leg varicose package and perforans veins was noted.

DISCUSSIONS

The perturbation of the equilibrium between MMPs and TIMPs was proposed as a mechanism for the accumulation of extracellular matrix, in varicose veins (Woodside et al., 2003).
MMPs family includes until present 26 enzymes, 24 being identified by their codifying genes (Visse, Nagase, 2003). Based on substrate specificity, sequence similarity and domain organization, vertebrate MMPs are included into six groups: collagenases, gelatinases, stromelisines, matrylisines, membranar-type MMP and other types (Visse, Nagase, 2003, Marchenko et al., 2003). MMP-2 (A gelatinase or IV collagenase of 72 kDA) and MMP-9 (B gelatinase or 92 KDa gelatinase) contain three modules of fibronectin type II composing a compact domain of collagen binding (Visse, Nagase, 2003).
Gelatinases degrade collagen type IV, V, VII, X, XI, and XIV, gelatine, elastine, proteic core of proteoglycans, myelinic basic protein, fibronectin, fibrillin-1, and TNF-α and IL-1b precursors. Through these multiple substrates, gelatinases have a major role in degrading extracellular matrix and remodeling, in implantation processes and wound healing (Visse, Nagase, 2003).
TIMPs family has four members (TIMP-1, 2, 3, 4), with natural inhibitory role of MMPs activity. TIMPs (21-29 kDa) have an amino- terminal end (125 amino acids) and a carboxy-terminal end (65 amino acids), each containing three disulfidic constant bindings; N-terminal end is folded as a separate unit and is capable of MMP inhibition (Folgueras et al., 2004).
TIMP-1 inhibits the activity of the majority MMP, excepting MT1-MMP and MMP-2. TIMP-1 and TIMP-2 are able to bind to the hemopexin domain of latent MMP-9 and MMP-2. TIMP-1 inactivates MMP-1, MMP-2, MMP-3 and MMP-9. TIMP-1 is able to produce complexes with proMMP-9, blocking its enzymatic activity (Folgueras et al., 2004).
TIMP-1 expression is stimulated by growth factors, cytokines and phorbolic esters (Hanemaaijer et al., 1993, Maier et al., 1993, Richards, Agro, 1994; Chua et al., 1996, Borden, Heller, 1997).
TIMP-2 inhibits the MMP activity, excepting MMP-9 (Folgueras et al., 2004) and pro-MMP-2. TIMP-2 is constitutively expressed paired with MMP-2 (Richards, Agro, 1994, Borden, Heller, 1997).
The literature review regarding MMPs and TIMPs study in varicose disease permitted an evaluation of the results obtained by several researchers interested in this subject. A special mention needs the existence of only few studies centered on immunohistochemical investigations, and the results are prone to controversy.
High levels of MMP-1 and TIMPs, and MMP-2 low levels are reported in the saphenous-femorale comparing to control veins (Parra et al, 1998). Different regional differences are reported in the matrix metalloproteinases components and in MMP-1 and MMP-13 activity, between proximal and distal segments of varicose veins (Shireman et al., 1996, Gillespie et al., 2002). Other differences were obtained between the distribution of MMP-1 and MMP-9 (Woodside et al, 2003), both normal and varicose veins expressing MMPs in endothelial cells, smooth muscular fibers and fibroblasts, but the endothelial reaction in adventiceal microvasculature, in normal veins, being qualitatively and quantitatively stronger.
Plasminogen activators seem to play an important role in MMP activation resulting in fibrosis of the venous wall and varicose dilation; these findings need supplementary researches (Shireman et al., 1996, Badier-Commander et al., 2000, Kosugi et al., 2003).

Several studies on advanced stages of the varicose disease, complicated with varicose ulcer, reveal that MMP-2, MMP-9 and TIMP-1 had high levels in the exudate of patients diagnosed with varicose veins in comparison to traumatic wounds (Wysocki et al, 1993, Bullen et al 1995, Weckroth et al, 1996).

The analysis of skin biopsies offers supplementary data on the increase of MMP-1, MMP-2 and TIMP-1 in patients with lipodermatosclerosis comparative to normal skin (Herouy et al, 1998). Patients diagnosed with varicose veins have an increased expression of MMP-2, positive reactions for the MMP extracellular promoter, type 1 and 2 membranar receptors of MMP in dermis and in perivascular regions of the varicose veins (Herouy et al, 2000a, Herouy et al., 2000b). Although, in patients in CEAP IV stages, comparative to CEAP V, no significant differences could be identified between the levels and activity of MMP-1, MMP-2, MMP-9 and TIMP-1 (Saito et al., 2001).

For both investigated groups, our results revealed evident differences between the two investigated MMPs. In a small number of analyzed cases we evidentiated an inconstant MMP-2 presence. On the other hand, MMP-9 was expressed in all cases, in a parallel manner to TIMP-1.

Thus, we do not consider MMP-2 expression a characteristic of the matrix remodeling process in the affected venous wall. Oppositely, MMP-9 involvement is certain, its intervention being counterbalanced by that of its specific inhibitor, TIMP-1, evidently stronger in immunohistochemical expression. In this direction, MMP-9 and TIMP-1 distribution offers important data. Our data indicated MMP-9 presence in endothelial layer, while TIMP-1 was absent in the same location. This finding sustains the role of endothelial lesion, a possible explanation being the fact that MMPs synthesis by endothelial cells is not inhibited by the specific inhibitor.

In the medial tunic, MMP-9 was primarily evidentiated around muscular cells, and TIMP-1 in neoformation capillaries. The different location suggests a parallel implication of the two cellular populations, the production of MMPs by smooth muscular cells being controlled by TIMP-1 production by endothelial cells. In adventicia, MMP-9 and TIMP-1 production was present both in *vasa vasorum*, and in fibroblastic-type cells. Consequently, we appreciate that in adventicia, the matrix modifications are more influenced by the MMPs and TIMPs action, maintaining equilibrium between them. Despite this, as a result of MMPs action, adventiceal connective tissue will evolve towards a fibrous transformation.

Comparing our results for the group 1 and group 2 respectively, in simple and complicated varices, resulted the absence of a significant difference between MMPs and TIMPs profiles. Thus, the hypothesis that morphological (tissular, cellular, and molecular) modifications can not be individualized and precisely correlated to the clinical stage of the disease (Saito et al., 2001).

There are few comparative studies, in the literature, on different regions of the venous system of the inferior limb with venous insuffiency (Shireman et al., 1996), with results that report low levels of MMPs in the inferior (proximal) region, comparing to the superior (distal) region. Oppositely, our data sustain the absence of major differences in the MMPs and TIMPs profile between the cross, internal saphene vein, leg varicose package and perforans veins. Similar modifications in MMPs and TIMPs profile, in different segments, indicate the complete involvement of the venous system that may be correlated to genetic predisposition.

Despite differences between various studies, MMPs and TIMPs production alterations may modulate, in the early stages of venous insuffiency, tissue remodeling and results, in advanced cases, in tissular fibrosis.

CONCLUSIONS

Although our data indicate a MMP-9 and TIMP-1 involvement in matrix remodeling, the complete profile of the family members and their action and regulation mechanisms are still unknown. The intercellular and molecular relation in chronic venous insuffiency deciphering opens the pathway for new treatment methods and prevention.

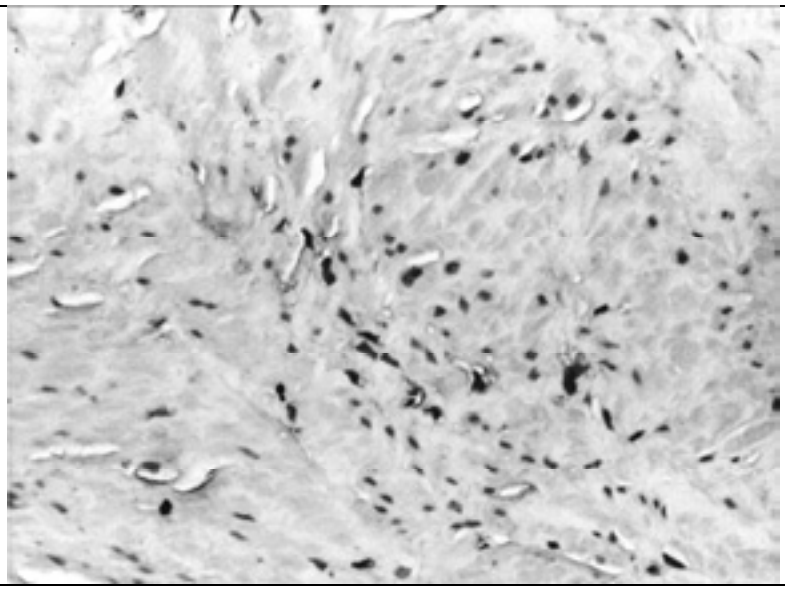

Fig. 1. Varicose package. Positive MMP-9 immunohistochemical staining in interstitium and in neoangiogenesis intramural capillaries (x20)

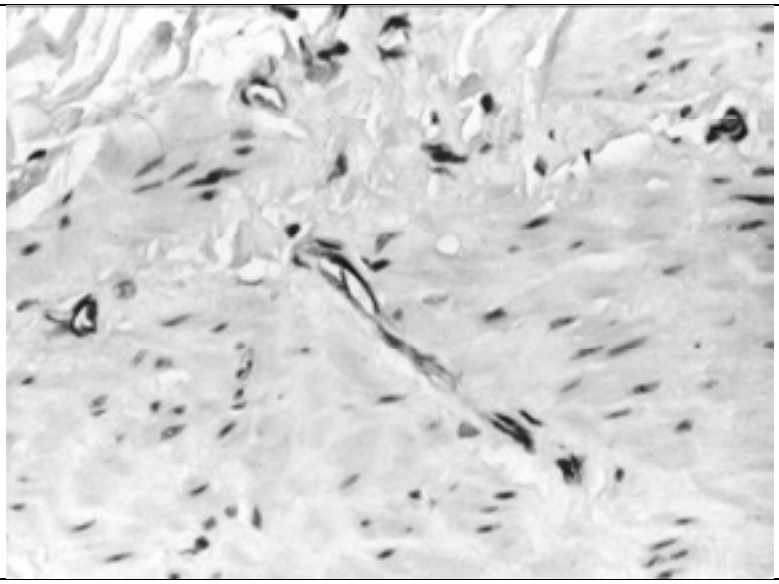

Fig. 2. Varicose package. MMP-2 positive immunohistochemical stain in vasa vasorum (x 20)

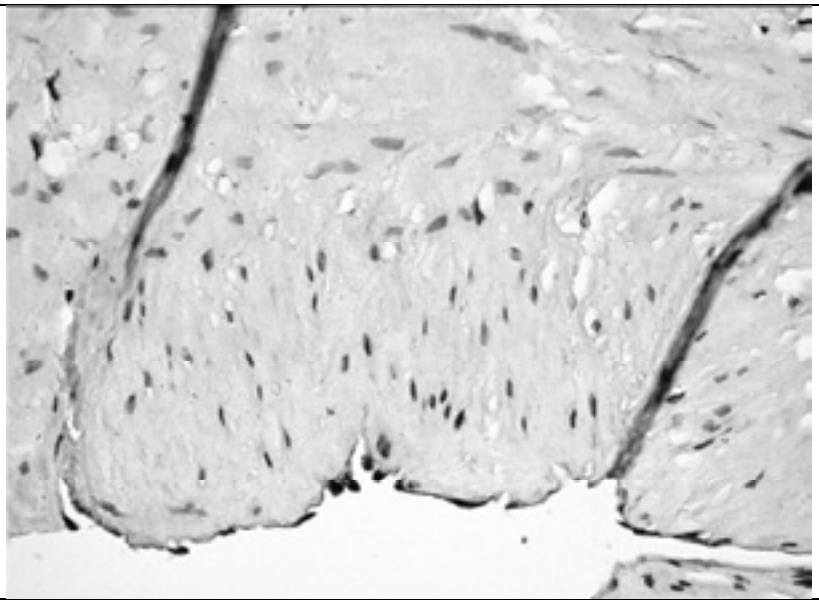

Fig. 3. Varicose package. Saphene cross. Discontinuous, weakly positive MMP-9 immunohistochemical staining in venous endothelium (x40)

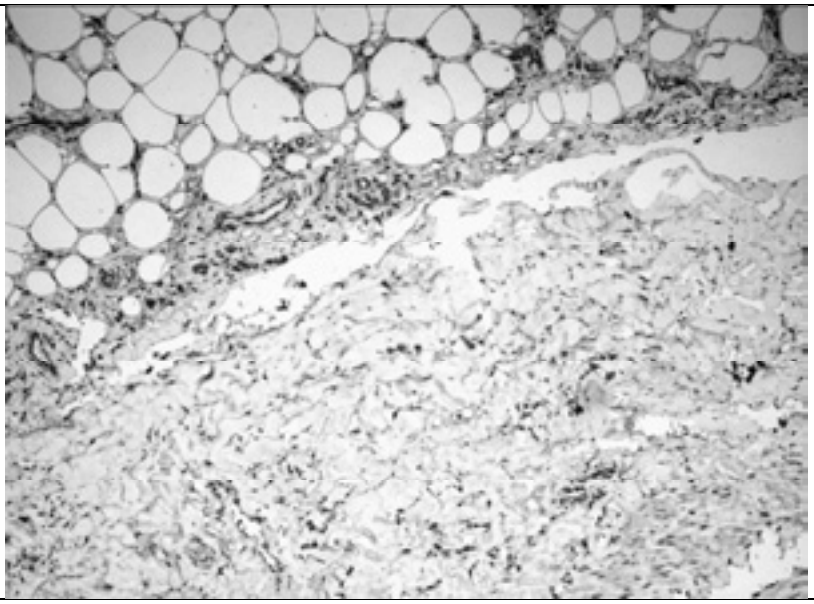

Fig. 4. Saphene cross. Positive TIMP-1 immunohistochemical staining in vasa vasorum and in capillaries (x10)

REFERENCES

Badier-Commander, C., Verbeuren, T., Lebard, C., Michel, J. B., Jacob, M. P., 2000, *J. Pathol*, 192: 105-112.
Borden, P., Heller, R. A., 1997, Crit Rev Eukaryot Gene Exp, 7: 159-178.
Bullen, E. C., Longaker, M. T., Updike, D. L., Benton, R., Ladin, D., Hou, Z., Howard, E. W., 1995, *J. Invest. Dermatol.*, 104: 236–240.
Chua, C. C., Hamdy, R. C., Chua, B. H., 1996, Biochim. *Biophys. Acta*, 1311: 175-180.
Folgueras, A. R., Pendas, A. M., Sanchez, L. M., Lopez-Otin, C., 2004, *Int. J. Dev. Biol*., 48: 411-424.
Gillespie, D. L., Patel, A., Fileta, B., Chang, A., Barnes, S., Flagg, A., Kidwell, M., Villavicencio, J. L., Rich, N. M., 2002, *J. Surg. Res.*, 106: 233–238.
Hanemaaijer, R., Koolwijk, P., le Clercq, L., de Vree, W. J., van Hinsbergh, V. W., 1993, *Biochem. J*., 296: 803-809.
Herouy, Y., May, A. E., Pornschlegel, G., Stetter, C., Grenz H., Preissner, K. T., Schöpf, E., Norgauer, J., Vanscheidt, W., 1998, *J. Invest. Dermatol*., 111: 822–827.
Herouy, Y., Trefzer, D., Hellstern, M. O., Stark, G. B., Wanscheidt, W., Schöpf, E., Norgauer, J., 2000, *Br. J. Dermatol*., 143: 930–936.
Herouy, Y., Trefzer, D., Zimpfer, U., Schöpf, E., Wanscheidt, W., Norgauer, J., 2000, *Eur. J. Dermatol*., 9: 173–180.
Johnson, J. L., van Eys, G. J., Angelini, G. D., George, S. J., 2001, *Artereoscler. Thromb. Vasc. Biol.*, 2: 1146-1152.
Kockx, M. M., Knaapen, M. W., Bortier, H. E., Cromheeke, K. M., Boutherin-Falson, O., Finet., M., 1998, *Angiology*, 49: 871-877.
Kosugi, I., Urayama, H., Kasashima, F., Ohtake, H., Watanabe, Y., 2003, *Ann. Vasc. Surg*., 17: 234–238.
Li, Y. Y., McTiernan, C. F., Feldman, A. M., 2000, *Cardiovasc. Res*., 46: 214-224.
Maier, R., Ganu, V., Lotz, M., 1993, *J. Biol. Chem*., 268: 21527-21532.
Marchenko, G. N., Marchenko, N. D., Strongin, A. Y., 2003, *Biochem. J.*, 372: 503–515.
Michiels, C., Arnould, T., Thibaut-Vercruyssen, R., Bouaziz, N., Janssens, D., Remacle, J., 1997, *Int. Angiol.*, 16: 134-141.
Porto, L. C., da Silveira, P. R., de Carvalho, J. J., Panico, M. D., 1995, *Angiology*, 46: 243-249.

Richards, C. D., Agro, A., 1994, *Cytokine*, 6: 40-47.
Rose, S. S., Ahmed, A., 1986, *J. Cardiovasc. Surg.*, 27: 534-543.
Saito, S., Trovato, M. J., You, R., Lal, B. K., Fasehun, F., Padberg, F. T., Hobson, R. W. 2nd, Durán, W. N, Pappas, P. J., 2001, *J. Vasc. Surg.*, 34: 930–938.
Sansilvestri-Morel, P., Nonotte, I., Fournet-Bourguignon, M. P., Rupin, A., Fabiani, J. N., Verbeuren, T. J., Vanhoutte, P. M., 1998, *J. Vasc. Res.*, 35: 115-123.
Shireman, P. K., McCarthy, W. J., Pearce, W. H., Shively, V. P., Cipollone, M., Kwaan, H. C., Yao, J. S., 1996, *J. Vasc. Surg.*, 24: 719-724.
Travers, J. P., Brookes, C. E., Evans, J., Baker, D. M., Kent, C., Makin, G. S., Mayhew, T. M., 1996, *Eur. J. Vasc. Endovasc. Surg.*, 11: 230-237.
Vaalmo, M., Mattila, L., Johansson, N., Kariniemi, A. L., Karjalainen-Lindsberg, M. L., Kähäri, V. M., Saarialho-Kere, U., 1997, *J. Invest. Dermatol.*, 109: 96-99.
Venturi, M., Bonavina, L., Annoni, F., Colombo, L., Butera, C., Peracchia A, Mussini, E., 1996, *J. Surg. Res.*, 60: 245-248.
Visse, R., Nagase, H., 2003, *Circulation Res.*, 92: 827-839.
Weckroth, M., Vaheri, A., Lauharanta, J., Sorsa, T., Konttinen, Y. T., 1996, *J. Invest. Dermatol.*, 106: 1119–1124.
Woodside KJ, Hu M, Burke A., Murakami, M, Pounds, L. L., Killewich, L. A., Daller, J. A., Hunter, G. C., 2003, *J. Vasc. Surg.*, 38: 162-169.
Wysocki, A. B., Staiano-Coico, L., Grinell, F., 1993, *J. Invest. Dermatol.*, 101: 64–68.

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