Oxidized LDL induce hsp70 expression in human smooth muscle cells

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Abstract Heat shock protein 70 (hsp70) has been detected in atherosclerotic lesions, in which endothelial cells and smooth muscle cells are involved. In a previous report we showed that Ox-LDL, a causal factor in atherosclerosis, could induce hsp70 expression in cultured human endothelial cells [Zhu et al. B.B.R.C 1994, 200: 389]. Here, with immunofluorescence and immunoblotting techniques, we show that Ox-LDL are capable of inducing hsp70 expression also in human smooth muscle cells, and that this induction is dependent on cell density and on the concentration of Ox-LDL. The induced expression of hsp70 was higher in human umbilical vein smooth muscle cells than in a human smooth muscle cell line. Conversely, Ox-LDL was cytotoxic to both types of cells, more so to the human smooth muscle cell line. These observations indicate that Ox-LDL may be a stress responsible for hsp70 expression in atherosclerotic plaques and the presence of hsp70 in plaques may be a useful marker for continuous oxidative damage in the arterial wall.

Key words: Human smooth cell; Heat shock protein 70; Oxidized-LDL; Cytotoxicity; Atherosclerosis

1. Introduction

Hyperthermia and other toxic stimuli result in an increased production of stress proteins, or heat shock proteins (hsps). Hsps afford protection against proteotoxic factors by maintaining cellular proteins in their functional conformation [1] These inducible polypeptides have constitutive cognates, which, in physiological conditions, ensure the correct folding and targeting of newly synthesized proteins [1]. Hsps are classified into several groups on the basis of molecular mass, and the 70 kDa hsps (hsp70) are the subject of extensive research because they are often highly induced in stressed cells and are thought to represent a basic feature of cell ability to cope with adverse conditions [2]. We previously reported that Ox-LDL, an important etiological factor of atherosclerosis, trigger the expression of the inducible form of hsp70 in cultured human endothelial cells [3]. Furthermore an increased expression of hsp70 has been observed in human atherosclerotic plaques [4–6]. Since smooth muscle cells are primarily involved in the formation of atheroma, we addressed the question whether Ox-LDL may represent a stress able to induce human smooth muscle cells to express hsp70.

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Abbreviations: Ac-LDL, acetylated low density lipoproteins; BrdU, bromodeoxyuridine; BSA, bovine serum albumine; Hsps, heat shock proteins; LDL, low density lipoprotein; HUVSMC, human umbelical vein smooth muscle cells; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PBST, phosphate-buffered saline + 0.1% Tween 20.

2. Materials and methods

2.1. Cells

Human umbilical vein smooth muscle cells (HUVSMC) were isolated as described [7]. In brief, the umbilical cord vein was cannulated at both ends and rinsed with lactated Ringer's (Hartmann's) solution. Firstly, endothelial cells were removed by 0.1% collagenase incubation (10–20 min). Smooth muscle cells were then obtained by further treatment of the vein with 0.2% collagenase for 30–60 min. Cells were collected by low speed centrifugation and grown at 37°C in a humidified atmosphere of 5% CO_2 in MEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM tricine buffer and 1% (v/v) non-essential amino acid solution. Cells were used between the 1st and 3rd passage. Smooth muscle cells were identified on the basis of their morphology and by mean of a monoclonal antibody specific for α-actin, the actin isoform typical of smooth muscle cells [8]. A-617 SMC myocytes, a smooth muscle cell line derived from human femoral artery (kindly provided by Dr. G. Gabbiani, Geneva), were grown in the same conditions.

2.2. Lipoproteins

LDL were isolated by sequential ultracentrifugation from freshly drawn human plasma, containing 0.01% EDTA (w/v), [9]. Ac-LDL were prepared by repeated additions of acetic anhydride, according to Basu et al. [10], and Ox-LDL were prepared in the presence of 20 μM Cu^{2+}, as described [11].

2.3. Immunofluorescence

Smooth muscle cells were grown on glass coverslips in 24-well culture plates and incubated with lipoproteins in fresh medium without FCS. Immunofluorescence staining procedure was the same as described [3]. Specific monoclonal antibodies to hsp70 (1:200) were used, followed by biotinylated anti mouse IgG (1:250) and by fluoresceinated streptavidin (1:100). The mouse monoclonal antibody specific for the inducible form of hsp70 (C92F3A-5) was purchased from StressGen (Canada). Mouse monoclonal antibodies specific for α-actin and β-actin (cytoplasmic isoform) were purchased from Amersham (England).

2.4. Immunoblotting

Cells were incubated with Ox-LDL at different concentrations for 12 h, then lysed in TRIS buffer containing SDS (3%) and PMSF (1 mM); protein was determined according to Lowry et al. [12], and equal amounts of protein from the different samples were subjected to SDS-PAGE on a 10% gel, after the addition of β-mercaptoethanol (2%), glycerol and bromophenol blue. Electrophoresed proteins were transferred onto a nitrocellulose membrane using a trans blot cell (Bio-Rad, USA) [13]. The membrane was incubated with PBS/3% BSA (blocking buffer) for 1 h at 25°C, then with PBS/0.1% Tween-20 (PBST) containing anti hsp70 mouse monoclonal antibody (C92F3A-5) for 1 h. After 3 washes with PBST, the membrane was incubated for 1 h in PBST containing a secondary antibody (goat anti-mouse IgG conjugated with peroxidase, Amersham, 1:2000). The membrane was washed in PBST and the immunocomplex was detected by an enhanced chemiluminescence method (ECL, Amersham).

2.5. Cell viability

Cell viability was evaluated by the MTT test [14]. Cells (30,000/well)

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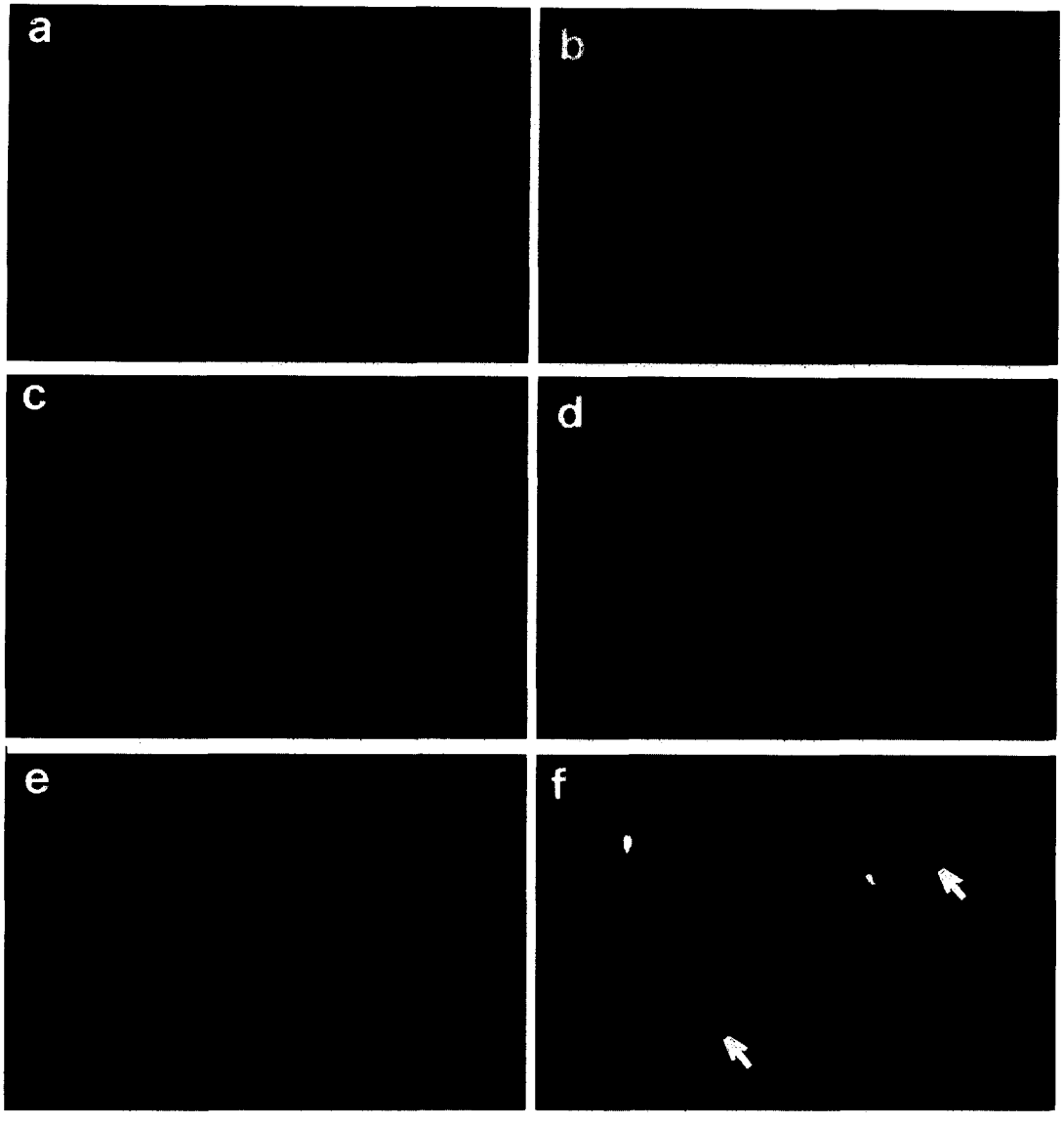

Fig. 1. Hsp70 induction by Ox-LDL in human umbilical vein smooth muscle cells (HUVSMC). Cells incubated without lipoproteins, as a control (a, ×1350); cells incubated with native LDL (200 μg/ml) for 12 h (b, ×1350); cells were incubated with Ox-LDL (200 μg/ml) for 12 h (c, ×350); cells were incubated with Ox-LDL plus cycloheximide (4 μg/ml) for 12 h (d, ×1350); confluent cells incubated with Ox-LDL for 12 h (e, ×350); confluent cells were considered with a Tefllon policeman, allowed to recover for 24 h and incubated with Ox-LDL for 12 h (f, ×350); panel (f) depicts both cells healing the lesion (left), displaying strong hsp70 staining, and confluent cells (right), displaying very faint staining. Arrows indicate the edge of the lesion. For experimental details refer to section 2.

in 12-well culture plates were incubated for 12 h in serum-free medium containing Ox-LDL (200 μg protein/ml), then incubated for 3 h in phenol red free-RPMI containing MTT (1 mg/ml). Isopropanol was used to dissolve transformed MTT and the absorbance at 560 nm was measured using an automatic plate reader (Titertek Multiscan 2). Cell viability was expressed as percent ratio over viable control cells, incubated in the presence of BSA (200 μg/ml).

3. Results

HUVSMC showed almost no staining when probed with the anti hsp70 antibody after an incubation in lipoprotein-free medium (Fig. 1a) or in medium containing native LDL (Fig. 1b). When non confluent HUVSMC were incubated with Ox-LDL (200 μg protein/ml) for 12 h, there was an intense, cytoplasmic staining revealing the expression of hsp70 (Fig. 1c); when the

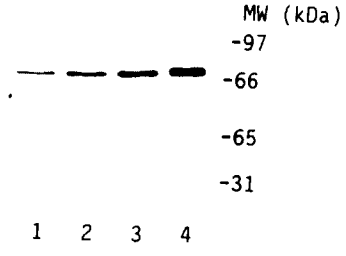

Fig. 2. Immunoblotting analysis of hsp70 in HUVSMC. Proliferating cells in sparse culture were incubated in the absence (lane 1) or in the presence of Ox-LDL (100, 200, 400 μg/ml, lanes 2–4) for 12 h. Cellular proteins (15 μg/lane) were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The inducible form of hsp70 was detected by mean of a specific antibody, as described in section 2.

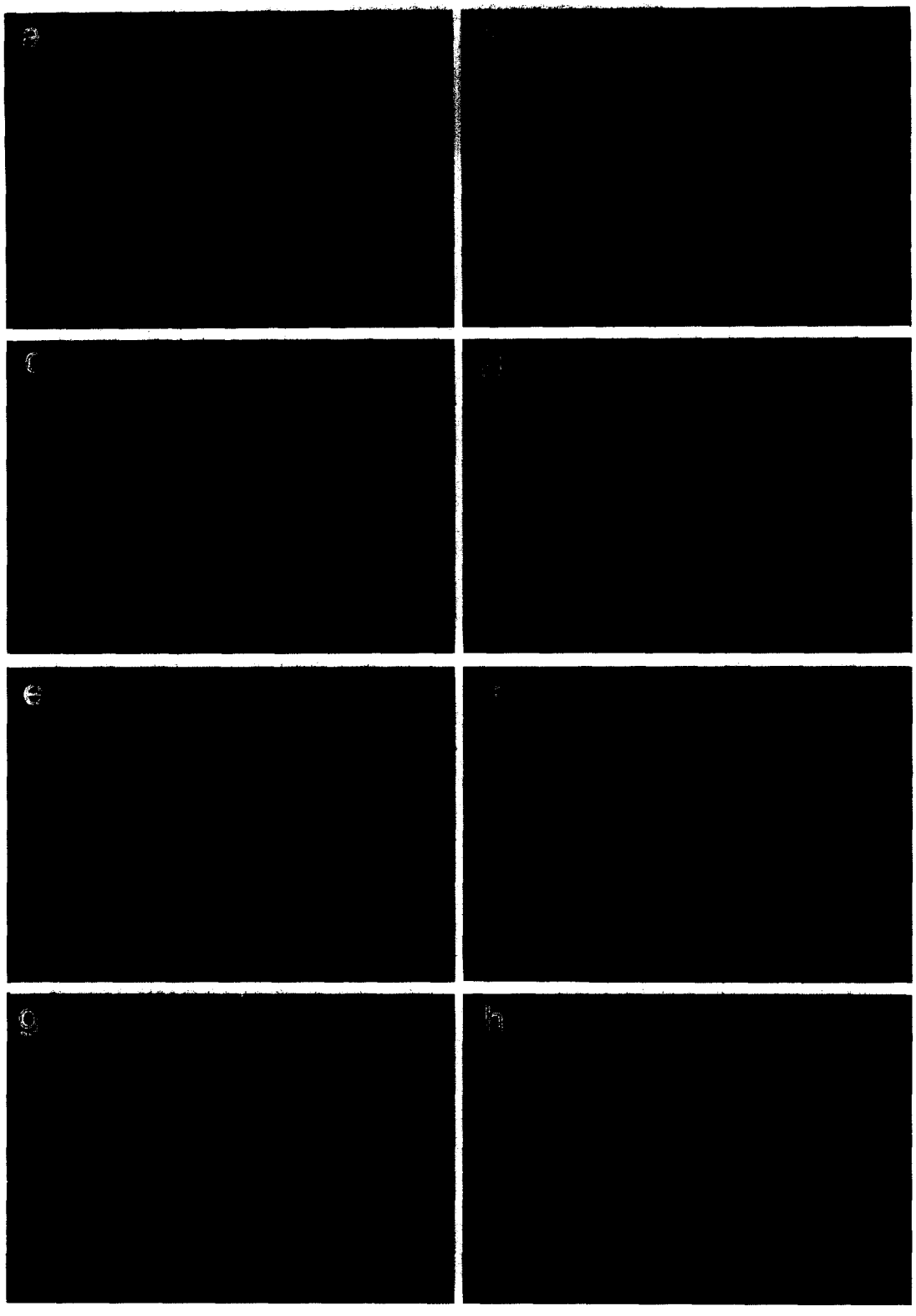

Fig. 3. Hsp70 induction by Ox-LDL and staining for α- and β-actin isoforms in HUVSMC (c,d,e,g) and in the A-617 SMC line (a,b,f,h). A-617 SMC line incubated with no lipoproteins, as a control (a, ×1350) and with Ox-LDL (400 μg/ml) for 12 h (b, ×1350); HUVSMC incubated with no lipoproteins, as a control (c, ×1350) and with Ox-LDL (400 μg/ml) for 12 h (d, ×1350); HUVSMC and A-617 SMC line probed with anti-α-actin antibody (e,f, ×1350); HUVSMC and A-617 SMC line probed with anti-β-actin antibody, (g,h, ×1350). For experimental details refer to section 2.

cells were incubated with Ox-LDL plus cycloheximide (4 μg/ml), a protein systhesis inhibitor, the expression of hsp70 was suppressed (Fig. 1d). Thus, hsp70 induction by Ox-LDL was dependent on protein systhesis. As we observed with human endothelial cells [3], the induction of hsp70 expression by OxLDL in confluent HUVSMC was much lower than in non-

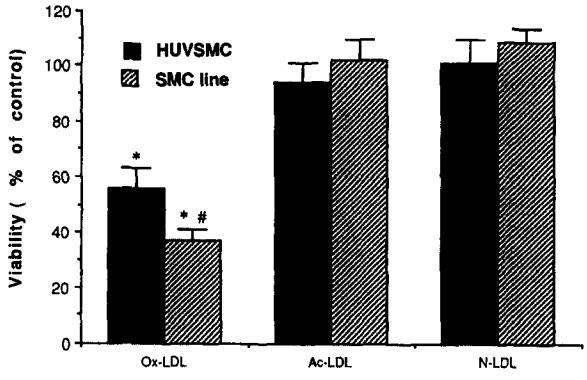

Fig. 4. Ox-LDL cytotoxicity to human smooth muscle cells (HUVSMC and the A-617 SMC line). Sparsely grown cells (30,000 cells/well) were incubated with Ox-LDL, Ac-LDL, native LDL (200 μg/ml) for 24 h. Viability was determined by the MTT test, as described in section 2, and expressed as percent of control cells, incubated in medium containing BSA (μg/ml). Values are the mean ± S.D. of 3 separate determinations. $^{*}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. Ox-LDL group of HUVSMC.

confluent cells (compare fig 1c and Fig. 1e). In order to understand why sparse and confluent cells have different responses to Ox-LDL, we performed a wounding experiment. A confluent culture of HUVSMC was wounded with a Teflon policeman and cells were allowed to recover in fresh medium for 24 h. Starting at this time, cells were incubated with Ox-LDL (400 μg/ml) for 12 h and subsequently stained for hsp70. Only cells healing the lesion displayed hsp70 staining, while staining of confluent cells was very faint (Fig. 1f). This result indicated that actively dividing or/and migrating cells, involved in wound repair, as sparse cells are likely to be, were more sensitive to Ox-LDL than relatively quiescent and stationary cells. Furthermore, immunoblotting results also indicated that the hsp70 expression was highly inducible in sparse cells (not shown) and the extent of expression was dependent on the concentration of Ox-LDL during the challenge (Fig. 2).

In order to confirm the above observations with smooth muscle cells of different origin, we performed immunofluorescence experiments with the A-617 smooth muscle cell line derived from human femoral artery. However, only a weak induction of hsp70 was observed when the cells were incubated with Ox-LDL up to 400 μg/ml (Fig. 3a,b). In the same experimental conditions, HUVSMC displayed very strong staining for hsp70 (Fig. 3c,d). Upon staining for smooth muscle α-actin, HUVSMC showed an intense fluorescence, with well organized α-actin bundles, while the A-617 SMC line only showed a faint staining (Fig. 3e,f). On the contrary, upon staining for β-actin HUVSMC showed weak staining and the A-617 SMC line displayed a relatively strong fluorescence (Fig. 3g,h). The results of an MTT test showed that, when cells were in sparse culture, Ox-LDL were toxic to both HUVSMC and, to a larger extent, to the A-617 SMC line (Fig. 4).

4. Discussion

De novo synthesis of stress proteins is generally regarded as a defense response triggered by toxic environmental conditions [1], and cytoprotection provided by hsps appears to be relevant in several pathologies [15]. Hsps may have a role also in atherosclerosis, since increased expression of hsp70 has been observed in human atherosclerotic lesions [4–6]. Recently, we reported that Ox-LDL, a cytotoxic lipoprotein, induce the expression of hsp70 in cultured human endothelial cells; we speculated that, in vivo, the presence of Ox-LDL might induce the expression of hsp70, possibly a cytoprotective response of arterial wall cells [3]. Oxidized LDL are thought to be a causal factor in atherosclerosis, and they have been detected in human and animal lesions [16]. In vitro, and most likely in vivo, Ox-LDL can damage smooth muscle cells, a major component of fibrous plaques in atherosclerotic lesions. Toxicity to smooth muscle cells is believed to represent an important atherogenic factor, since these cells are lost from deep- to mid-intimal locations during the development of the lipid-rich core of atherosclerotic plaques [17]. The finding that Ox-LDL induce the expression of hsp70 in cultured A-617 SMC as well as in endothelial cells reinforces the hypothesis that these lipoproteins may trigger a defense response in cells of the arterial wall. This may confer a relative resistance to repeated challengles sustained (driven) by Ox-LDL or, possibly, other toxic. Indeed, survival of smooth muscle cells to cytotoxic conditions is significantly improved by heat shock [6] or by a pretreatment with exogenous hsp70 [6,18]. Preliminary data in our laboratory also indicate that induction of hsp70 expression in human endothelial cells confers protection against highly toxic and potentially lethal concentrations of Ox-LDL (unpublished observations). As it occurs in several diseases [19] oxidation appears to be involved in atherosclerosis [20] and the use of antioxidants has proved beneficial for the prevention or the correction of experimental [21] and clinical [22] atherosclerosis. The oxidative burst associated with ischemia/reperfusion increases the expression of hsp70 [23] and, accordingly, induction of hsp70 expression protects from ischemia/reperfusion injury [24]. The balance between proatherogenic insults (i.e. Ox-LDL toxicity) and the ability of the cell to cope with them (i.e. stress response) can influence the evolution of atherosclerotic lesions. In the presence of Ox-LDL, confluent HUVSMC express much less hsp70 than sparse ones. Accordingly, wounding experiment indicated that actively dividing or/and migrating smooth muscle cells (i.e. sparse cells) are much more sensitive to Ox-LDL than relatively stationary cells (i.e. confluent cells). Consistent results were obtained in wounded endothelial cells that had been labeled with BrdU before the addition of Ox-LDL. Double immunofluorescence staining indicated in fact that the majority of cells at the boundary of the injury area expressed hsp70 and were BrdU positive (data not shown). That is, actively cycling cells are most sensitive to the stress.

It is generally accepted that smooth muscle cell proliferation and migration from the arterial tunica media into the intima are essential steps in the development of atheromatous plaque [25–28]. The present results may be relevant to the understanding of smooth muscle cell response to injury in proatherosclerotic events.

The comparison between HUVSMC and the A-617 SMC line offered some interesting information. Differently from HUVSMC, the SMC line showed very weak hsp70 expression even at a high concentration of Ox-LDL, although Ox-LDL were cytotoxic to both types of cell. Interestingly, HUVSMC were damaged by Ox-LDL less than the A-617 SMC line was. We do not know whether this may relate to the higher expression of hsp70, a cytoprotective protein, in HUVSMC.

HUVSMC, which were used between the 1st and 3rd passage after isolation, displayed a strong staining for α-actin and low staining for β-actin, while the permanent A-617 SMC line displayed a faint staining for α-actin and a relatively strong staining for β-actin. Since phenotypic changes in smooth muscle cells are associated with changes in the relative expression of actin isoforms [29,30]. This might be a very important issue since development of atherosclerotic plaques is characterized by the transition of SMC phenotype from a contractile to a synthetic one [31–33]. Further experiments are needed to confirm this hypothesis.

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References

[1] Hightower, L.E. (1991) Cell 66, 191–197.
[2] Feige, U. and Polla, B.S. (1994) Experientia 50, 979–986.
[3] Zhu, W., Roma, P., Pellegatta, F. and Catapano, A.L. (1994) Biochem. Biophys. Res. Commun. 200, 389–394.
[4] Berberian, P.A., Myers, W., Tytell, M. and Bond, M.G. (1990) Am. J. Pathol. 136, 71–80.
[5] Johnson, A.D., Berberian, P.A., Tytell, M. and Bond, M.G. (1993) Exp. Mol. Pathol. 58, 155–168.
[6] Johnson, A.D., Berberian, P.A., Tytell, M. and Bond, M.G. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 27–36.
[7] Gimbrone, M.A. and Cotran, R.S. (1975) Lab. Invest. 33:16–27.
[8] Skalli, O., Ropraz, P., Trezciak, A., Benzonana, G., Gillessen, D. and Gabbiani, G. (1986) J. Cell Biol. 103, 2787–2796.
[9] Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345–1353.
[10] Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3178–3182.
[11] Roma, P., Bernini, F., Fogliatto, R., Bertulli, S.B., Negri, S., Fumagalli, R. and Catapano, A.L. (1992) J. Lipid Res. 33, 819–829.
[12] Lowry, O. H., Rosebrough, N. J. Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Towbin, M., Staehlin, T., and Gordon, J. (1979). Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[14] Denizot, F. and Lang, R. (1986) J. Immunol. Methods 89, 271–277.
[15] Minowada, G. and Welch, W.J. (1995) J. Clin. Invest. 95, 3–12.
[16] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L., (1989) N. Engl. J. Med. 320, 915–924.
[17] Hughes, H., Mathews, B. and Guyton, J.R. (1994) Artherioscler. Thromb. 14, 1177–1185.
[18] Johnson, A.D., Berberian, P.A. and Bond, M.G. (1990) Atherosclerosis 84, 111–119.
[19] Haliwell, B. and Gutteridge, M.C. (1985) Oxford, England, Claredon Press.
[20] Lyons, T.J. (1993) Am. J. Cardiol. 71, 26B–31B.
[21] Wojcicki, J., Rozewicka, L., Barcew-Wiszniewska, B., Samochowiec, L., Juzwiak, S., Kadlubowska, D., Tustanowski, S. and
Juzyszyn, Z. (1991) Atherosclerosis 87, 9–16.
[22] Gaziano, J.M., Manson, J.E. and Hennekens, C.H. (1994) In: Natural Antioxidants in Human Health and Disease (B. Frei ed.), Academic Press, pp. 387–409.
[23] Marber, M.S., Walker, J.M., Latchman, D.S. and Yellon, D.M. (1994) J. Clin. Invest. 93, 1087–1094.
[24] Liu, X., Engelman, R.M., Moraru, I.I., Lu, D., Nath, D., Rouson, J.A., Flack, J.E., Deaton, D.W., Manlik, N. and Das, D.K. (1992) Circulation 86 (Suppl II), 358–363.
[25] Ross, R. and Glomset, J.A. (1976) N. Engl. J. Med. 295, 369–377.
[26] Ross, R. and Glomset, J.A. (1976) N. Engl. J. Med. 295, 420–425.
[27] Haust, M.D. (1974) Adv. Exp. Med. Biol. 43, 35–57.
[28] Benditt, E.P. and Gown, A.M. (1980) Int. Rev. Exp. Pathol. 21, 55–118.
[29] Gabbiani, G., Kocher, O. and Bloom, W.S. (1984) J. Clin. Invest. 73, 148–152.
[30] Kocher, O., Skalli, O., Bloom, W.S. and Gabbiani, G. (1984) Lab. Invest. 50, 645–652.
[31] Chamley-Campbell, J., Campbell, G.R. and Ross, R. (1979) Physiol. Rev. 59, 1–61.
[32] Schwartz, S.M., Campbell, G.R. and Campbell, J.H. (1986) Circ. Res. 58, 427–444.
[33] Ross, R. (1993) Nature 362, 801–809.