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OXIDIZED-LDL INDUCE THE EXPRESSION OF HEAT SHOCK PROTEIN 70 IN HUMAN ENDOTHELIAL CELLS

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Summary: Heat shock proteins are detectable in human atherosclerotic plaques, especially in endothelial cells. In this report we show by immunofluorescence that incubation "in vitro" with OxLDL is a stress capable of inducing the expression of heat shock protein 70 in both the EAhy-926 cell line and human umbilical vein endothelial cells (HUVEC). This induction was parallel to the cytotoxicity of oxidized LDL as determined by [^{3}H]adenine release. When cells were confluent, however, both effects were greatly reduced. We speculate that induction of hsp70 is related to the citotoxicity of oxidized LDL and that the detection of heat shock proteins in human atherosclerotic plaques is a further indication for the presence "in vivo" of oxidized LDL. These observations may be relevant to the understanding of endothelial response to injury in proatherosclerotic events.

Different kinds of environmental assault can induce cells, from the simplest bacterium to the most highly differentiated neuron, to express stress proteins, known as heat shock proteins (hsps). Hsps can prevent damage to other cellular proteins and allow denatured proteins to reacquire their native conformation (1). Recently, hsp65 and hsp70 have been detected in human atherosclerotic plaques, particularly in endothelial cells and macrophages, and T lymphocytes specifically recognizing hsps have been detected in areas of atherosclerotic lesions (2-5). These observations suggest that hsps may play a role in atherosclerosis. Evidence exist for the presence of oxidized low density lipoprotein (OxLDL) in atherosclerotic lesions from man and experimental animals, furthermore OxLDL are toxic to cultured endothelial cells and may cause endothelial cell injury in vivo (6). We therefore addressed the question whether OxLDL may represent a stress able to induce human

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Abbreviations: hsp, heat shock protein; LDL, low density lipoprotein; AcLDL, acetylated low density lipoprotein; OxLDL, oxidized low density lipoprotein; MEM, minimal essential medium; FCS, foetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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endothelial cells to express hsp70, the hsp which is most often highly induced in stressed cells and represents a fundamental feature of a cell ability to cope with "stress" conditions (3).

MATERIALS AND METHODS

Cells

EAhy-926, a permanent hybrid cell line between human umbilical vein endothelial cells and human lung carcinoma cells (7), were used as a model for human endothelial cells. Cells were maintained in standard conditions in MEM (Gibco, Madison, WI) with the addition of 10% FCS. 1% HAT was added to the culture medium to prevent the overgrowth of epithelial cells. Human umbilical vein endothelial cells (HUVEC) were cultured in M199 with the addition of 20% FCS, endothelial cell growth factor and heparin (90 μg/ml). Cells were used for experiments before the 5th passage.

Lipoproteins

LDL were isolated from freshly drawn human plasma, containing 0.01% EDTA (w/v), by sequential ultracentrifugation (8). AcLDL were prepared according to Basu (9) and Ox-LDL were prepared in the presence of 20 μM Cu^{++}, as described (10).

Immunofluorescence

Endothelial cells were incubated at 37°C for various times in fresh medium without FCS and with or without lipoproteins, or heat shocked at 45° C (15'), fixed with 3% paraformaldehyde at room temperature (RT) (15'), washed with PBS and permeabilized with 0.5% Triton X-100 on ice (3'). After blocking the nonspecific staining by an incubation in PBS with 1% BSA and 5% normal goat serum (1 h), cells were incubated in a 1:200 dilution of a mouse monoclonal antibody specific for the inducible form of hsp70 (C92F3A-5, StressGen, Canada) (RT, 1h) washed with PBST (0.05% Tween-20 in PBS) and incubated with biotinylated Anti-mouse IgG 1:250 (Amersham) (RT, 1 h) washed with PBST, then incubated with straptavidin-fluorescein 1:100 (Amersham, U.K.) (RT, 30') washed with PBST, mounted on microscopy slides and observed with a fluorescence microscope (Zeiss Aksioscope, Germany). Kodal Gold II (400 ASA) films were used for photographs.

[^{3}H]-Adenine release

Endothelial cell damage was estimated by [^{3}H]-adenine release as described (11). Briefly, cells grown in 12-well plates (Falcon, U.S.A.) were incubated at 37°C (2h) in medium without FCS containing 1 μCi/ml [^{3}H]-adenine (995.3 GBq/mmol, Dupont, U.S.A.). The labeled cells were rinsed four times with PBS to remove free [^{3}H]-adenine. Cells were incubated in medium containing lipoproteins or BSA (200 μg/ml). At the end of the incubation the medium was immediately centrifuged at 1300 g (5'). A portion of the supernatant was subjected to radioisotopic counting. The cells were solubilized with 0.25 N NaOH and the radioactivity of one aliquot was also counted. [^{3}H]-adenine release was calculated as the ratio of radioactivity in the supernatant to the total activity initially taken up by the cells.

RESULTS

EAhy-926 cells displayed weak staining (fig. 1a) when probed with the anti hsp70 antibody in the absence of any lipoprotein (0 h): this may be due to the low level expression of hsp70 that has been reported for other cell types in the absence of stress (12). After 7 h of incubation with 200 μg/ml OxLDL an intense, cytoplasmic staining was observed in non confluent cells (fig. 1b). However, when cells were

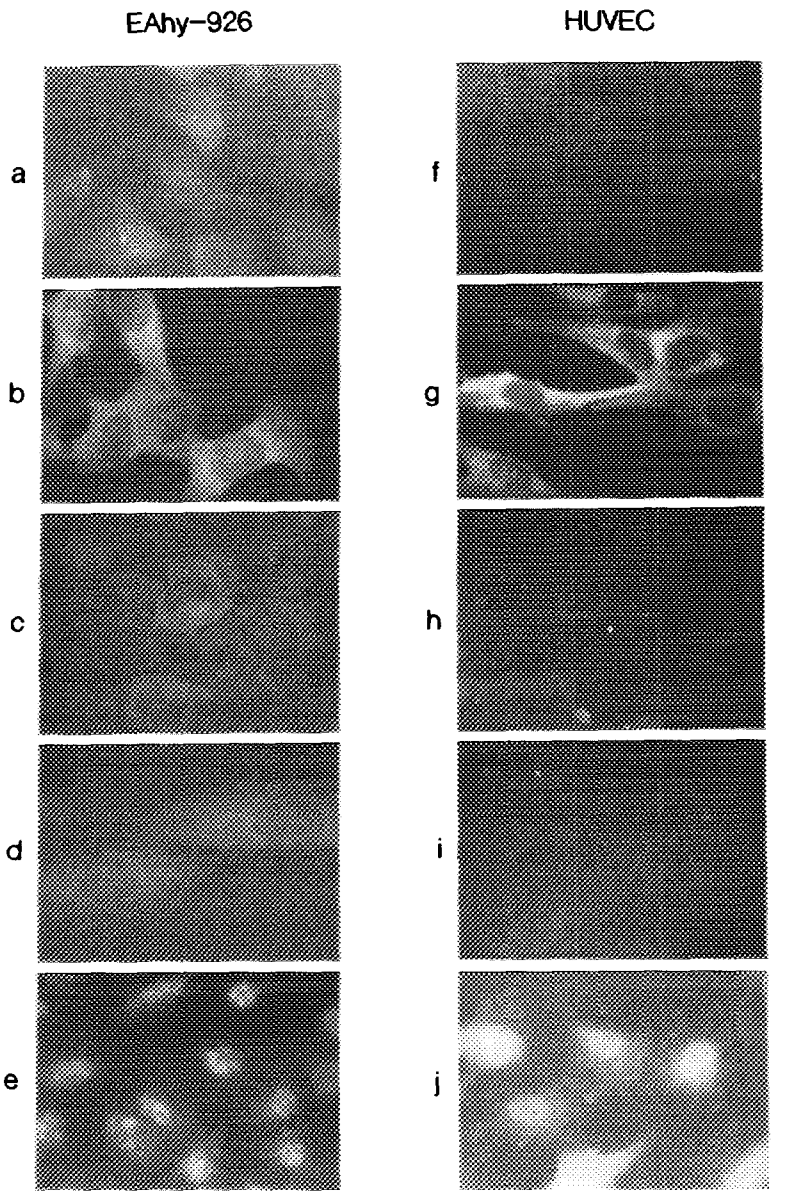

Figure 1. Sparse and confluent monolayers of EAhy-926 (**a**-**e**) and HUVEC (**f**-**j**) were incubated at 37° C for the indicated times in fresh medium without FCS, containing OxLDL or LDL (200 μg/ml), or heat shocked at 45° C for 15'. Cells were then processed for immunofluorescence with anti hsp70, as described in Materials and Methods, examined with a fluorescence microscope and photographed. Panels refer to: control incubation, no lipoproteins (**a**, **f**); incubation with OxLDL (**b**, **g**: sparse monolayers and **c**, **h**: confluent monolayers); incubation with LDL (**d**, **i**); heat shock (**e**, **j**).

fully confluent there was little induction of hsp70 (fig 1c). Staining of cells incubated with native LDL was very weak (fig 1d). Staining of heat- shocked cells, shown as a positive control, indicates that, after thermic stress, hsp 70 mainly localizes in the nucleus (fig. 1e), as reported for other cells (13).

Table I. Effect of cell density on OxLDL cytotoxicity

	[^{3}H]-Adenine Release (%)			
	Ox-LDL	Ac-LDL	LDL	BSA
Sparse cells	52.4±7.10	20.2±0.85	21.3±0.42	21.3±0.10
Confluent cells	20.0±1.74	19.5±0.57	22.6±1.07	21.1±0.49

[^{3}H]-adenine labeled EAhy-926 cells were incubated for 24 h at 37° C with either OxLDL, AcLDL, LDL or BSA (200 µg/ml) and [^{3}H]-adenine release was evaluated as described in Materials and Methods. Values are the mean±SE of 3 dishes and are representative of 4 separate experiments.

Since EAhy-926 are hybrid cells, and may not be fully representative of human endothelial cells, we wondered wether similar results would be obtained with HUVEC. In control conditions the expression of hsp70 in HUVEC was even lower than in EAhy (fig. 1f). At 7 h of incubation with OxLDL, the cytoplasm was heavily stained (fig. 1g), as observed for EAhy-926. Moreover, like EAhy-926, confluent HUVEC displayed almost no staining after 7 h of incubation with OxLDL (fig. 1h). Incubation with native-LDL did not induce expression of hsp70 (fig. 1i). As observed for EAhy-926, in heat shocked HUVEC hsp70 localized mainly within the nucleus (fig. 1j). Since hsp synthesis is a response to toxic stimuli we aimed at verifying the linkage between the increase in hsp expression and OxLDL-cytotoxicity. To test OxLDL cytotoxicity, [^{3}H]-adenine release from labeled EAhy-926 was evaluated. During incubation of sparse cells with OxLDL, [^{3}H]-adenine release was more than twofold that observed after incubation with either AcLDL, native LDL, or BSA (table I). When confluent cells were incubated with OxLDL [^{3}H]-adenine release was the same as that observed in the presence of the other lipoproteins or BSA (table I). Similar results were obtained in HUVEC (data not shown).

DISCUSSION

"Ex novo" synthesis of hsps is a defense response triggered in cells by a variety of noxious stimuli (1). Since increased expression of members of the heat shock protein family has been observed in atherosclerotic lesions, it is conceivable that hsps play a role in cellular events related to this pathology. Oxidation of LDL appears to be a key event in atherosclerotic lesion formation (6,10) and oxidized LDL are toxic to cells (14). Therefore, we asked the question whether exposure to chemically oxidized LDL (OxLDL), a model of oxidatively modified lipoproteins

may sustain hsp synthesis. Our attention was focused on endothelial cells because endothelial lining is a major target during vessel wall injury (14); furthemore, endothelial cells can oxidize LDL (15) and oxidative events are likely to occur in the subendothelial space. In both EAhy-926 and HUVEC, increased expression of hsp70, was evident after a 7 hour incubation with OxLDL. Interestingly, the effect of this treatment was different from that of heat shock: in fact, heat shock and recovery, resulted in nuclear accumulation of hsp70 (13), while during incubation with OxLDL (200 μg/ml) newly expressed hsp70 was essentially located in the cytoplasm. At longer incubation times, however, translocation of hsp70 from the cytoplasm to the nucleus was observed in both EAhy cells and HUVEC (data not shown).

Stress proteins initially localize in sites of major injury (16). In heat-shocked cells this occurs in the nucleus. Conversely when cells take up OxLDL, cytoplasm is the first site of injury and, with prolonged incubation, OxLDL toxicity eventually affects the nucleus, where hsp may then accumulate. OxLDL can trigger a stress response also in other cell types, like mouse peritoneal macrophages, where increased expression of hsp32 and heme oxygenase has been reported (17).

The present study underscores a difference between sparse and confluent cells in the susceptibility to OxLDL cytotoxicity. Since protein denaturation is a prerequisite for the synthesis of hsps (18), it is reasonable to speculate that the resistance of confluent cells to OxLDL toxicity may result in little expression of hsp70. This observation may bear significance to the "in vivo" action of OxLDL on the arterial wall. Sparse endothelial cells, which are actively proliferating "in vitro", may be compared with an injured endothelium involved in wound repair, a characteristic of early atherosclerotic lesions. "In vivo", cytoprotection afforded by stress proteins may allow cells higher chances to survive cytotoxic events, possibly the exposure to OxLDL; the observed expression of hsp by endothelial cells in areas of atherosclerosis is in agreement with this hypothesis (4). Understanding the hsp function in this context requires further "in vitro" and "in vivo" studies, and the hybrid cell line EAhy-926 appears to be a suitable model of human endothelium for future investigation.

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