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Low density lipoprotein activates monocytes to express tumor necrosis factor

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We analyzed the effect of acetylated low density lipoprotein (aLDL) incubation on tumor necrosis factor (TNF) mRNA and protein expression in isolated resting human monocytes in serum free DMEM. TNF mRNA expression was about one third that of PMA and was dose dependent. The maximum stimulatory effect on TNF mRNA was at 250 μg/ml, while 500 μg/ml induced downregulation. The maximum stimulatory effect occurred at 6 hours, and by 24 hours TNF mRNA expression returned to the resting state. Acetyl LDL also induced the expression of immunoreactive TNF, reaching a sevenfold maximum above control at 12 hours following a 6 hour exposure period. The results suggest that aLDL is a potent stimulator of TNF expression in resting monocytes. This mechanism may be operational in atheroma evolution.

Monocyte; Tumor necrosis factor; Low density lipoprotein; Gene expression

1. INTRODUCTION

Monocytes are thought to participate in atherogenesis, both as foam cells [1,2] and as a source of growth factors and cytokines [3]. One such cytokine is tumor necrosis factor (TNF) which can cause cell proliferation [4,5], angiogenesis [6] and cell necrosis [7]. We have previously detected TNF in human atheroma by immunohistochemistry [8] and by in situ hybridization [9]. Resting monocytes, however, do not express the TNF gene [10]. Its expression is induced by factors such as phorbol esters, e.g. PMA [11], LPS [12] or even by the cell isolation process [11]. Since LDL is a major lipid that accumulates in Mo [13] and SMC [14] of the atherosclerotic lesion, and is also a general cellular activator [15], we hypothesized that LDL may trigger TNF production in Mo. Consequently, the goals of this study were (i) to determine if LDL activates Mo to express TNF, and (ii) to determine the dose dependence and time kinetics of Mo activation.

2. MATERIALS AND METHODS

2.1. *Reagents and cells*

LDL, HDL, PMA, Histopaque, PMX B, Na acetate, and lipoprotein-deficient FCS were obtained from Sigma; RPMI 1640, DMEM and tissue culture dishes from Gibco; tissue culture chambers from LAB-TEK; TNF ELISA kit (Biokine) from T Cell Sciences; anti human rTNF alpha monoclonal antibody was a gift of Dr M. Narachi (Amgen); Limulus amebocyte lysate endotoxin assay (Pyrotell) from Associates of Cape Cod; Ilford K5 nuclear emulsion from Ilford.

Monocytes were obtained from the buffy coat of healthy human donors with normal lipoprotein profiles. The cells were separated on Histopaque discontinuous gradient [16]. Cells, at a concentration of $0.5–2.8 \times 10^6$/dish, were incubated in RPMI 1640 supplemented with 10% lipoprotein-deficient FCS, and were allowed to attach for 18 h in 35 mm plastic dishes in 5% CO_2 at 37°C. Cells were >93% viable (Trypan blue exclusion) and were proven to be >90% monocytes by non-specific esterase staining. At the end of the attachment period the medium was replaced with serum free DMEM for the rest of the experiments. As monocyte-derived macrophages express only few native LDL receptors and internalize only minimal lipoprotein [17], we used chemically modified (acetylated) LDL which is taken up more rapidly by way of distinct, scavenger receptors [17,18]. Cells were incubated with either DMEM alone (control), with PMA (positive control) or with aLDL (experimental group). LDL was acetylated by saturated sodium acetate and the success of acetylation was verified by the typical enhanced electrophoretic mobility [18]. We also incubated Mo in some experiments with equivalent concentration of native LDL and HDL.

2.2. *RNA extraction and Northern analysis*

Total cellular RNA was isolated by an acid guanidinium thiocyanate-phenol-chloroform extraction technique [19] and was analyzed by Northern blot [20]. The cDNA probe was a human 800 bp *Eco*RI cDNA fragment from Genentech, San Francisco, CA [21]. Autoradiograms were read on a semiautomatic densitometer (E-C Apparatus Corporation).

2.3. *Histological techniques*

Mo were fixed in the culturing chamnbers by 10% neutral buffered formalin. For immunohistochemical staining antihuman rTNA alpha monoclonal antibody and peroxidase labelled double antibody technique was used as described earlier [8]. In situ hybridization on freshly fixed Mo was carried out with the TNF cDNA probe as described earlier [9]. TNF concentration in the supernatant was measured with a commercial ELISA kit [22] which showed good correlation with L 929 Bioassay.

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Abbreviations: TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; LDL, low density lipoprotein; aLDL, acetylated LDL; HDL, high density lipoprotein; DMEM, Dulbecco's Modified Eagle Medium; ELISA, Enzyme Linked Immunoabsorbant Assay; PMX B, Polymyxin B; FCS, fetal calf serum; LPS, lipopolysaccharide; Mo, monocytes; SMC, smooth muscle cells

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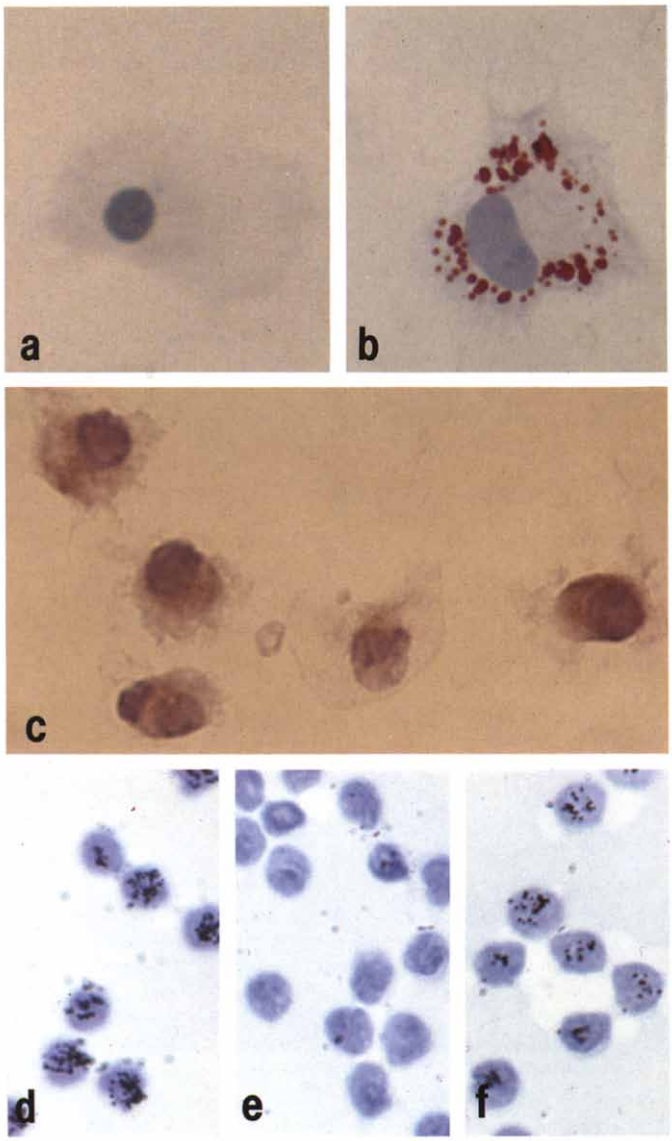
2.4. *Screening for endotoxin contamination*

All components of the incubating system were screened for endotoxin by chromogenic limulus amoebocyte lysate assay. We used only materials with endotoxin levels less than 40 pg/ml in these experiments. In some instances we ran parallel experiments with or without LPS antagonist PMX B (10 μg/ml) [11] and compared the results.

3. RESULTS

3.1. *Morphological finding*

Oil red O staining showed heavy perinuclear lipid accumulation in monocytes exposed to aLDL, but not in control cells (Fig. 1a,b). In situ hybridization revealed accumulation of silver grains exclusively over the monocytes indicating TNF mRNA expression (Fig. 1c, d,e). Immunohistochemical staining revealed diffuse cytoplasmic TNF positivity (Fig. 1f).



3.2. *Kinetics of TNF mRNA accumulation*

Northern blot analysis revealed induction of TNF mRNA expression in monocytes incubated with both PMA and aLDL. The aLDL induced signal was about one third of that induced by PMA (Fig. 2 and Table I). Acetyl LDL induction of TNF mRNA expression was dose dependent: peak level was achieved at 250 μg/ml aLDL concentration, while at 500 μg/ml concentration the expression of TNF mRNA significantly decreased (Fig. 3 and Table I). When Mo were incubated continuously with 250 μg/ml aLDL, the peak level of TNF mRNA expression occurred at 6 h and returned to baseline within 24 h (Fig. 4 and Table I).

3.3. *Secretion of TNF by aLDL incubated monocytes*

The immunoreactive TNF concentration in the incubation media was 3-fold greater in the Mo incubated with aLDL than in those incubated with DMEm alone after 6 h incubation. Native LDL and HDL did not induce TNF expression. The peak TNF concentration (7-fold greater than the control level) was reached at 6 h after removal of aLDL. After 24 h TNF was undetectable in the supernatant of both the treated and the control Mo (Fig. 5).

4. DISCUSSION

In this study we report for the first time that acetylated LDL incubation induces the expression of TNF mRNA in human Mo. The increased mRNA expression was accompanied by production of immunoreactive TNF. The strength of TNF mRNA induction

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Fig. 1. Panel a and b: Oil red O staining of human blood Mo. Methylene blue nuclear staining. Panel a: one of the control Mo. They were allowed to attach in RPMI 1640 medium containing 10% lipoprotein deficient FCS for 18 h, then they were incubated in serum-free DMEM without aLDL. There is no cytoplasmic staining with Oil red O. Panel b: one of the aLDL incubated monocytes stained by oil red O. Following the attachment period they were incubated with 250 μg/ml aLDL for 6 h. There is intensive cytoplasmic staining with Oil red O indicating massive lipid uptake. Panel c: immunohistochemical staining for TNF. Mo incubated with 250 μg/ml aLDL for 6 h were stained using monoclonal antibody against rhTNF. α and peroxidase labelled double antibody technique. Cells show positive cytoplasmic brown staining. Panels d-f: in situ hybridization for TNF mRNA. Nuclear staining with hematoxylin. Control Mo were unstimulated (Panel e). Mo were stimulated with 50 ng/ml PMA for 4 h (Panel d) or were incubated with 250 μg/ml of aLDL for 6 h (Panel f). Cells were fixed in the tissue culture chambers with 10% neutral buffered formalin, then were digested by proteinase K for 15 min at 37°C and treated in acetic anhydride for 10 min at room temperature. We probed the cells with a human TNF cDNA probe (in 50% formamide, 2% dextrane sulphated, ds thymus DNA and EDTA) labelled with 2 Ci/ng probe [α-^{32}P]dCTP for 18 h at 37°C in a humidity chamber. Slides were washed with high stringency (max at 55°C in 0.1 SSC for 2 × 15 min). Slides were coated with Ilford K5D nuclear emulsion and were exposed for 3 days. Control Mo reveal only background level activity. After PMA and aLDL incubation there was an increase in TNF mRNA accumulation.

was one-third of that of PMA, one of the strongest known inducers of TNF gene expression [11].

The mechanism by which aLDL stimulates TNF expression is not clear. TNF production in monocytes is a complex process that involves both transcriptional and post-transcriptional control [10]. PMA, a tumor promoter seems to induce TNF production in monocytes through direct protein kinase C activation [23]. Acetyl LDL has recently been shown to be a general cellular activator which acts by increasing membrane receptor mediated phosphatidylinositol turnover [15,24] which in turn secondarily translocates (activates) protein kinase C [25]. Thus, aLDL could act earlier in the sequence of activation, while inducing TNF expression through a mechanism similar to PMA.

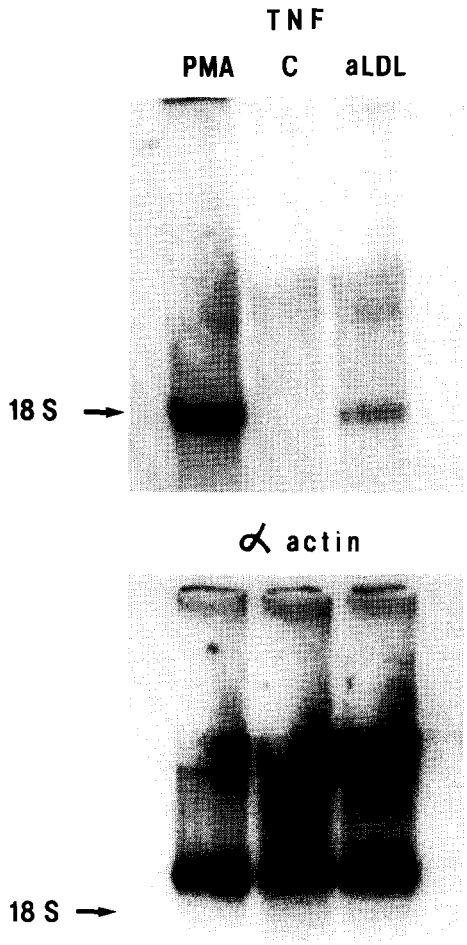

Fig. 2. Northern analysis of TNF mRNA of human Mo. Freshly prepared Mo were incubated in RPMI 1640 and 10% lipoprotein deficient FCS and allowed to attach for 18 h. The attached monocytes were then incubated either in serum-free DMEM alone (C) or in that containing 50 ng/ml PMA or 250 μg/ml aLDL. Total RNA was extracted and 20 μg RNA were run on each lane of an agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabelled TNF cDNA probe. In the control Mo incubated in DMEM alone there was no TNF mRNA expression while both PMA and aLDL induced TNF mRNA expression. Alpha actin probing showed no induction and indicates similar amounts of RNA on each lane.

Table I

Effect of aLDL on TNF mRNA. The values shown are the integrals of peaks determined by scanning autoradiograms in the linear range of the film with a semi-automatic densitometer. The amount of RNA/lane, labelling and exposure conditions were uniform within each group of experiments

	TNF	ACTIN
PMA	317,250	441,510
C	5,450	403,530
aLDL	86,430	451,075
50	101,340	
100	318,610	
250	273,910	
500	36,818	
8	518,880	
6	997,110	
12	790,790	
18	627,700	
24	2,957	
36	14,210	
48	10,902	

Acetyl LDL is taken up by scavenger receptors [17]. Since there was no other source of lipid in the serum-free medium, the heavy cytoplasmic lipid accumulation demonstrated by Oil red O stain suggests that the accumulated lipid was aLDL, although we did not specifically established the identity of the intracellular

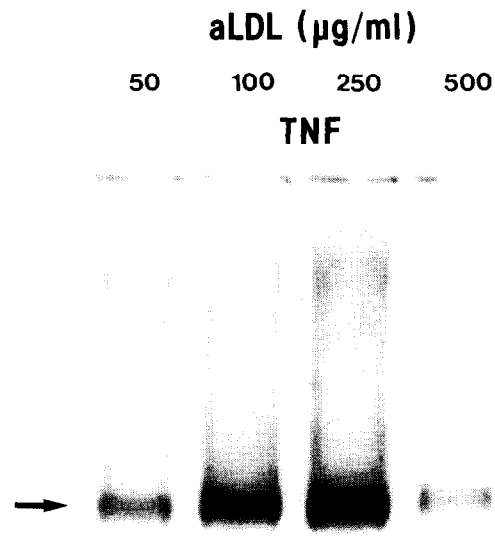

Fig. 3. Dose-dependence of the induction of TNF mRNA by aLDL. After the attachment period (see Fig. 2) Mo were incubated with the indicated concentrations of aLDL. Total RNA was extracted and probed as above. Maximal induction occurred at 250 μg/ml aLDL concentration, while at 500 μg/ml TNF mRNA seemed to be down-regulated.

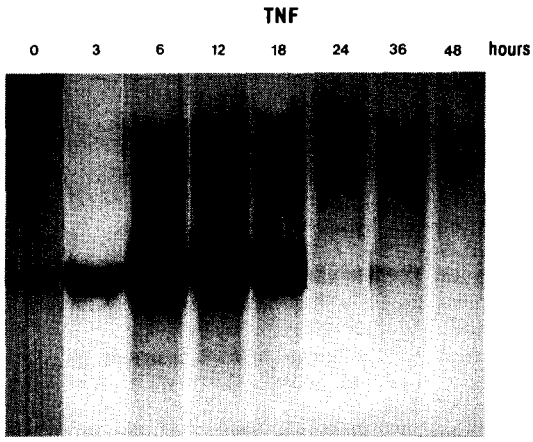

Fig. 4. Time kinetics of the induction of TNF mRNA by aLDL incubation in human Mo. After the attachment period Mo were incubated with 250 μg/ml aLDL. Following the indicated incubation periods total RNA was extracted and hybridized to TNF cDNA probes. The peak level of TNF mRNA expression was reached at 6 h of stimulation with aLDL; by 24 h TNF mRNA expression decreased to the steady state level.

lipid. Minimal cytoplasmic lipid accumulation was detected by Oil red O after incubation with native LDL and HDL. These lipids did not induce TNF expression although they can activate Mo, e.g. increasing β-glucuronidase secretion [26]. Thus, our data suggest that intracellular aLDL accumulation may cause TNF gene expression, although activation of the membrane-localized phosphatidylinositol system during the entry of aLDL is also a possibility. Presence of oxidatively modified LDL [27] and the functional integrity (including TNF production) of lipid-loaded macrophages [28] have recently been demonstrated.

The time course of TNF mRNA accumulation and protein secretion was consistent with transcriptional and translational activation. In spite of the continuous presence of aLDL in the media, the Mo TNF mRNA expression was transient, returning to the resting level in 24 h. To study the time course of TNF protein production, we removed the aLDL after 6 h of incubation and actually measured the production and decay of TNF in 6 h periods. TNF production peaked 6 h after removal, i.e., 12 h after initial exposure and became undetectable at 24 h. There was also transient TNF production detected in the control Mo. This was attributed to the transient activation of Mo during the isolation procedure.

Both LDL [13] and Mo [12] are involved in atherogenesis. Our study suggests one potentially important link between the two, through expression of TNF. The role of TNF and other cytokines in atherogenesis has not been established. Nevertheless, only atherosclerotic human arteries express TNF, and the amount of immunoreactive TNF increases as the atheroma becomes complicated [8]. Thus, it is possible that LDL uptake initiates production of growth factors and cytokines such as TNF and IL-1 that are mitogenic [4,5], angiogenic [6] and cytotoxic [7], leading to evolution of the atheroma from the fatty streaks through the proliferative lesion to vascularized, necrotic unstable atheroma.

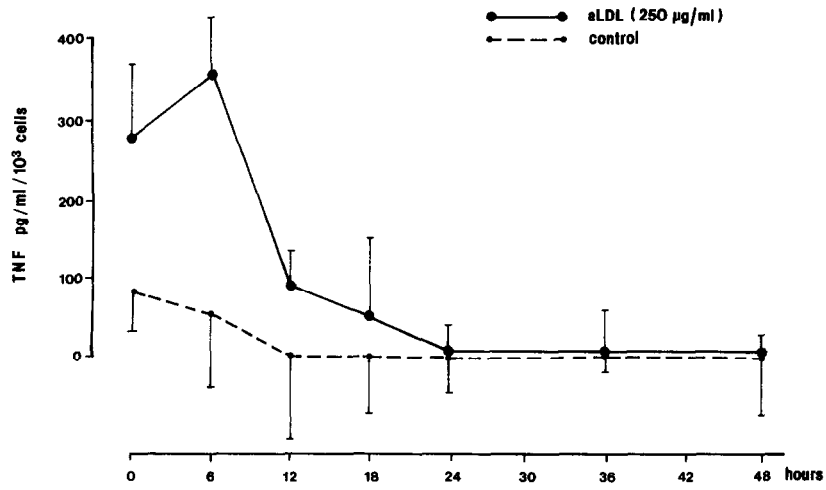

Fig. 5. Time kinetics of TNF protein production by human Mo measured by ELISA. After the attachment period Mo were incubated either in serum-free DMEM alone or in serum-free DMEM containing 250 μg/ml aLDL for 6 h. The first ELISA was performed at the end of this period (0 h). Then the medium was changed to fresh serum-free DMEM in both groups at the periods indicated on the abscissa. Data represent TNF production and decay by 1000 Mo within the indicated time intervals after a single 6 h stimulation with aLDL. Data shown are mean ± SEM of duplicates for 3 experiments. TNF production was higher at the end of the incubation with aLDL than with medium alone, and revealed further increase during the next 6 h period in spite of aLDL removal. Afterwards there was a sharp decrease in TNF production, such that by 24 h TNF was not detectable. In the control group there was a small and continuously decreasing TNF production.

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