Monocyte proliferation induced by modified serum is associated with endogenous M-CSF production: evidence for involvement of a signalling pathway via scavenger receptors

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Abstract. It was found that human serum stored for 2 months at 4°C (modified serum) induced monocyte proliferation and simultaneous macrophage colony stimulating factor (M-CSF) production by these cells *in vitro*. Cell number, estimated by DNA content, doubled after 10 days in culture in the presence of modified serum, while it decreased in culture with freshly thawed control serum. As the addition of more than 2.5 ng/ml of recombinant M-CSF significantly supported monocyte survival/proliferation, cells were cultured for 10 days in medium supplemented with control serum, and endogenous M-CSF production was investigated by enzyme-linked immunosorbent assay. M-CSF concentration in the supernatants was 15–30 ng/ml after 10 day in culture with modified serum, a level that might be sufficient for monocyte proliferation. The modified serum induced M-CSF from freshly isolated monocytes, while M-CSF was hardly detected in cultures supplemented with control serum. Assay for peroxidized lipid and agarose gel electrophoresis demonstrated that the modified serum contained more oxidized low density lipoproteins (LDL) than the control serum. Ligands of scavenger receptors, which are receptors for oxidized LDL, such as dextran sulphate, polyinosinic acid, heparin and acetylated LDL also significantly induced M-CSF production from human monocytes, although this was at levels below 2 ng/ml. These results indicate that serum modified by oxidation stimulates monocytes to produce M-CSF resulting in their proliferation, and that signalling via scavenger receptors is one of the mechanisms responsible for this induction of M-CSF.

INTRODUCTION

Macrophage colony-stimulating factor (M-CSF) is a glycoprotein that regulates the proliferation and function of mononuclear phagocytes (Stanley *et al.* 1983). M-CSF is produced by various types of cells including fibroblasts, vascular endothelial cells, stromal cells, and monocyte/macrophages, and is detected in both serum and urine (Ralph & Sampson-Johannes 1990). Since M-CSF acts specifically on monocyte/macrophage-lineage cells, it is plausible that these cells produce M-CSF to induce proliferation/survival and to regulate

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functional activities in an autocrine manner. M-CSF production from monocytes was reported to be augmented by treatment with vitamin D (Kaneki *et al.* 1994), phorbol 12-myristate 13-acetate (PMA) (Rambaldi, Young & Griffin 1987, Horiguchi, Sariban & Kufe 1988) or cytokines such as granulocyte/macrophage (GM)-CSF, tumour necrosis factor (TNF)- α and interferon (IFN)- γ (Horiguchi, Warren & Kufe 1987, Sherman *et al.* 1990, Yamada *et al.* 1991, Gruber *et al.* 1992b), by adherence to plastic substrate (Haskill *et al.* 1988), and by cellular maturation elicited by cultivation *in vitro* (Scheibenbogen & Andreessen 1991). In contrast, M-CSF production is suppressed by treatment with interleukin (IL)-4, IL-10 and prostaglandin E (Lee *et al.* 1990, Gruber *et al.* 1994, Hamilton 1994). Recent studies demonstrated that stimulation with antibodies against surface markers such as CD45, CD44 or lymphocyte function-associated antigen (LFA)-3 triggers M-CSF production by monocytes (Gruber & Gerrard 1992, Gruber *et al.* 1994).

M-CSF production is observed *in vivo* at the atherosclerotic intima of blood vessels, where increased numbers of macrophages and foam cells are observed (Rosenfeld *et al.* 1992). However, the physiological stimulator of M-CSF production at the atherosclerotic intima remains unclear. In this study, it was found that serum from prolonged storage is a potent inducer of monocyte proliferation through endogenous M-CSF content, and that modified low density lipoprotein (LDL) might play an important role in M-CSF induction by stimulation via scavenger receptors.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) (*Escherichia coli* 055:B5) (DIFCO, Detroit, MI, USA), PMA (Sigma, St Louis, MO, USA), acetylated LDL (Biomedical Technologies, Soughton, MA, USA), dextran sulphate (average molecular weight 500 kDa: Sigma), polyinosinic acid (poly(I)) (Sigma) and heparin (GIBCO, New York, NY, USA) were purchased. Pooled human serum Type AB was purchased from Sigma, and stored at -20°C until use. Modified serum was prepared by incubating human serum at 4°C for 2 months in sterilized condition. LPS concentrations in sera were determined using a toxinometer (Wako-jyunyaku, Osaka, Japan). Recombinant human M-CSF (Genetics Institute, Cambridge, MA, USA) was purified from culture supernatants of Chinese hamster ovary (CHO) cells transfected with human M-CSF cDNA.

Cell culture

Cells were prepared from peripheral blood of healthy volunteers by centrifugation on Ficoll and subsequent Percoll density gradients as described previously (Asakura *et al.* 1996). The centrifuged cells were greater than 80% monocytes as identified by nonspecific esterase staining (Mutokagaku, Tokyo, Japan) and by Wright Giemsa staining (Mutokagaku). Culture medium was prepared by mixing Ham's F12 with DMEM (1 : 1) (Nissui, Tokyo, Japan), and supplemented with 10% (v/v) human serum. Cells were suspended at $1 \times 10^6/\text{ml}$ in this medium, and cultured in 24-well plates (Costar, Cambridge, MA, USA). Scavenger receptor ligands were added at 1–100 $\mu\text{g}/\text{ml}$ for the induction of M-CSF. Media were changed twice a week, and cell numbers after 10 days in culture were estimated from DNA content assayed by an established method (Burton 1968) where 14 μg of DNA corresponding to 10^6 cells. A human monocytic cell line, THP-1, was obtained from the American Type Culture Collection (No.TIB202) and cultured in RPMI1640 (Nissui) supplemented with 10% fetal calf serum (Flow, Maclean, VA, USA).

Assays

M-CSF was measured by enzyme-linked immunosorbent assay (ELISA) (Hanamura *et al.* 1988). As human serum-supplemented medium contained 0.3 ng/ml of M-CSF, M-CSF concentration in supernatants was corrected by subtracting 0.3 ng/ml from the data.

Characterization of serum

Peroxidized lipid content was measured using a kit (Wako-jyunyaku). Changes in the electrophoretic mobility of LDL in sera were evaluated by 0.6% agarose gel electrophoresis in 0.05 M veronal buffer (pH 8.6), followed by staining with Oil red O (Merck, Darmstadt, Germany).

Statistics

The statistical significance of differences was determined by the paired Student's *t*-test for population data, and by the *F*-test followed by Student's *t*-test for individual data of triplicate cultures. A *P*-value of < 0.05 was considered statistically significant, but many of the significant values were *P* < 0.01.

RESULTS

Prior experiments indicated that human monocytes were maintained better *in vitro* when cultured with human sera that had been stored for prolonged periods at 4°C than with sera frozen until use. Thus, pooled human serum (Type AB) was left at 4°C for 2 months (modified serum) and used this for monocyte culture. Monocytes were prepared from peripheral blood of four donors and cultured in the presence of either modified serum or pooled serum frozen until use (control serum). As shown in Table 1, the cell numbers estimated by DNA content after 10 days were 2.5 to 19-fold higher in cultures supplemented with modified serum than in the control serum. In contrast to the control serum, the modified serum caused a twofold increase in the number of cells during culture for 10 days, indicating that the modified serum was inducing monocyte proliferation.

A prior previous study demonstrated that M-CSF supports monocyte survival in cultures supplemented with fetal calf serum (Asakura *et al.* 1996). Recombinant human M-CSF was added to the cultures with control serum, and cell numbers were estimated by DNA content after

Table 1. Modified serum induced monocyte proliferation and M-CSF production*

Monocyte source	Culture with control serum			Culture with modified serum**		
	DNA/well after 10 days (% of day 0)	M-CSF (ng/ml)	M-CSF (ng/μg DNA)	DNA/well after 10 days (% of day 0)	M-CSF (ng/ml)	M-CSF (ng/μg DNA)
Donor 1	64	1.8	0.20	160 (2.5)	15 (8.3)	0.67 (3.3)
Donor 2	44	0.8	0.13	260 (5.9)	30 (38)	0.82 (6.3)
Donor 3	12	0.6	0.36	230 (19)	19 (32)	0.59 (1.7)
Donor 4	17	0.6	0.25	240 (14)	26 (43)	0.77 (3.1)

*Monocytes from 4 donors were seeded at 1×10^6 cells/ml, and cultured in medium supplemented with human serum. Medium was changed twice a week. Cell numbers were estimated by DNA content, and the relative DNA content after 10 days from culture initiation (Day 0) are shown. M-CSF concentration in the supernatants was assayed after 10 days.

**Data are compared with those from control cultures, and the ratios are shown in parentheses.

Figure 1. M-CSF supports monocyte survival/proliferation during cultivation *in vitro*. Monocytes from five donors were suspended at 1×10^6 cells/ml in medium supplemented with the control serum, and cultured in the presence of 0–250 ng/ml of recombinant human M-CSF. After 10 days, surviving cells were estimated by DNA content, and presented as relative values to those at culture initiation. NS, not significant. **Statistically significant differences (paired *t*-test, $P < 0.01$).

10 days in culture (Figure 1). Monocytes from three of five donors showed an increase in cell number and the average numbers of cells from five donors were significantly increased by the addition of M-CSF at more than 2.5 ng/ml. Therefore, it was examined whether or not monocytes produced M-CSF in response to modified serum to induce proliferation in an autocrine manner during culture. M-CSF was assayed in the culture supernatants by ELISA. M-CSF concentration was 8 to 43-fold higher in cultures supplemented with modified serum than in those with control serum after 10 days (Table 1), which was sufficient for cell survival and/or proliferation (Figure 1). These results indicated that the modified serum induced M-CSF production and supported cell survival/proliferation of monocytes during 10 day in culture *in vitro*.

M-CSF production from freshly isolated monocytes is hardly detected, and is upregulated by differentiation from monocytes to macrophages, which occurs after culture for more than 6 days (Scheibenbogen & Andreessen 1991). Thus, it was examined whether the modified serum is able to induce M-CSF production by freshly isolated monocytes. Monocytes from four donors were prepared, and stimulated with the modified serum for 2 days. M-CSF concentration was assayed in the culture supernatants. While M-CSF production was negligible in control cultures, significant induction of M-CSF was detected in cultures supplemented with the modified serum (5–13 ng/ml) (Figure 2). These results indicated that the modified serum stimulated freshly isolated monocytes to produce M-CSF.

Figure 4. Modified serum induced M-CSF from freshly isolated monocytes. Freshly isolated monocytes from four donors were cultured at 1×10^6 cells/ml in medium supplemented with either modified (MS) or control serum (CS). After 2 days, M-CSF concentration in the supernatants was assayed. *Statistically significant differences (paired *t*-test, $P < 0.05$).

The modified serum had a more turbid appearance than the control serum. The LPS concentration of the modified serum was less than 10 pg/ml and not dissimilar to the control serum, and LPS had no effect on M-CSF production from monocytes (See Figure 4a). However, the modified serum contained more insoluble materials, and the peroxidized lipid concentration was 7-fold higher than that in the control serum (43 vs. 6.6 nmol malondialdehyde equivalents/ml). The electrophoretic mobility of the LDL in the modified serum was higher than that in the control serum, indicating that its LDL was modified by oxidation (Figure 3). These results suggested that oxidized LDL contained in the modified serum may induce M-CSF production from monocytes.

Monocytes from four donors were suspended in medium supplemented with control serum, and cultured for 2 days in the presence of acetylated LDL (100 μ g/ml), a modified LDL which acts on monocyte/macrophages by binding scavenger receptors similarly to oxidized LDL. M-CSF production was augmented by acetylated LDL in monocytes from three donors, while cells of one donor produced no detectable M-CSF regardless of addition of acetylated LDL (Figure 4a). The effect of acetylated LDL on M-CSF induction from monocytes from this donor was also examined, when culture supernatants was harvested at earlier times ($\bullet$ and $\bullet/\circ$ in Figures 4a and b, respectively). However, the concentration of M-CSF induced by acetylated LDL was below 2 ng/ml, lower than that induced by the modified serum (Figure 2). PMA (10^{-7} M) also enhanced M-CSF production in monocytes from three of the four donors, while LPS (1 μ g/ml) had no effect on M-CSF production in monocytes.

M-CSF concentration was measured before and from 3 to 72 h after acetylated LDL-stimulation (Figure 4b). M-CSF was not detected 3 h after stimulation, but was induced at 15 h independently of acetylated LDL. The early transient induction of M-CSF might have

Figure 3. Changes in electrophoretic mobility of LDL in sera during prolonged storage. Modified serum left at 4°C for 2 months and control serum were separated by 0.6% agarose gel electrophoresis, and LDL visualized by staining with Oil red O. LDL showed higher mobility in modified serum than that in control.

Figure 4. Induction of M-CSF production from monocytes. Freshly isolated monocytes from four donors were examined, and the data are shown with different symbols. (a) Monocytes at 1×10^6 cells/ml were incubated with or without acetylated LDL (100 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$) or PMA (10^{-7} M) for 2 days. M-CSF in the culture supernatants was assayed. (b) M-CSF accumulation in medium was monitored 3–72 h after cultivation with (closed symbol) or without (open symbol) 100 $\mu\text{g/ml}$ of acetylated LDL.

been caused by adherence to the plastic substrate (Haskill *et al.* 1988). M-CSF level decreased during the subsequent culture of monocytes when cells were cultured without acetylated LDL. However, M-CSF concentration was increased for up to 48 h after stimulation with acetylated LDL.

To confirm that acetylated LDL induces M-CSF from human monocytes, the effect of acetylated LDL on a human monocytic cell line, THP-1, was examined. THP-1 was cultured with or without the stimulants described above for 2 days, and M-CSF in the supernatants was assayed. Acetylated LDL significantly induced M-CSF similarly to PMA (Figure 5). LPS did not induce M-CSF production by this cell line.

Furthermore, the dose-response effect of acetylated LDL as well as other scavenger receptor ligands on M-CSF induction from monocytes was examined. Monocytes were incubated with or without 1–100 $\mu\text{g/ml}$ of acetylated LDL, dextran sulphate, poly(I) or heparin for 2 days. Cells of two of the four donors produced detectable amounts of M-CSF without any stimulation (Figure 6, open bar), and responded to stimulants better than the other cells. As shown in Figure 6, the most potent inducer of M-CSF from monocytes was dextran sulphate, which caused aggregation of the cells (data not shown). Heparin and poly(I) as well as acetylated LDL also significantly induced M-CSF. In contrast to the other ligands, poly(I) did not act on monocytes in a dose-dependent manner. Cells treated with

Figure 5. Induction of M-CSF production from THP-1. THP-1 cells at 1×10^6 cells/ml were incubated with or without acetylated LDL (100 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$) or PMA (10^{-7} M) for 2 days. M-CSF in the culture supernatants was assayed, and results are indicated as means $\pm$ SD of triplicate experiments. NS, not significant. * and **Significantly different from the control group (Student's *t*-test, $P < 0.05$ and < 0.01 , respectively).

Figure 6. Induction of M-CSF production from monocytes by scavenger receptor ligands. Freshly isolated monocytes from four donors were cultured with or without 1 (left), 10 (middle), 100 (right) $\mu\text{g}/\text{ml}$ of acetylated LDL, dextran sulphate, poly(I) or heparin for 2 days. M-CSF in the culture supernatants was assayed, and results are indicated as means $\pm$ SD of triplicate experiments. M-CSF concentration in the culture without any stimulants is shown as a control (open bar). * and **Significantly different from the control group (Student's *t*-test, $P < 0.05$ and < 0.01 , respectively).

more than 10 $\mu\text{g}/\text{ml}$ of poly(I) appeared dark, indicating a cytotoxic effect of poly(I) (data not shown). Although M-CSF was significantly induced by scavenger receptor ligands, M-CSF concentration was less than 2 ng/ml following stimulation with ligands at levels up to 100 $\mu\text{g}/\text{ml}$, which was not sufficient to induce monocyte proliferation (Figure 1). These results suggested that other stimulatory pathway(s) might be involved in rgw induction of M-CSF production in monocytes by the modified serum, in addition to that via scavenger receptors.

DISCUSSION

The present study found that serum stored for prolonged periods at 4°C induced monocyte proliferation, which might have been a result of endogenous M-CSF production. Haskill *et al.* (1988) reported that the adherence of monocytes to plastic substrate induces M-CSF mRNA expression, and the results also demonstrated that adherence caused transient production of M-CSF into the medium, which was, however, hardly detectable 2 days later. Little effect of LPS on M-CSF protein production from monocytes has been observed by several investigators (Becker, Devlin & Haskill 1989, Lee *et al.* 1990, Scheibenbogen *et al.* 1991, Gruber & Gerrard 1992) in accordance with observations in the present study. PMA has been reported to stimulate M-CSF mRNA expression in monocytes (Gruber & Gerrard 1992). Scheibenbogen *et al.* (1991) showed that M-CSF production is augmented during culture *in vitro*, which is consistent with the present data demonstrating that monocytes produced M-CSF *in vitro* after 10 days in culture, while little production of M-CSF was observed from freshly isolated monocytes.

The present study indicated that serum modified by prolonged storage at 4°C, and which contained oxidized LDL, induced M-CSF production from freshly isolated monocytes. Stimulatory effects of acetylated LDL on M-CSF production were also found. Rajavashisth *et al.* (1990) reported that LDL stored at 4°C for 4 months, i.e. minimally modified LDL, induces endothelial cells to express M-CSF. As the authors did not detect any effect of malondialdehyde-modified LDL, they concluded that minimally modified LDL elicits M-CSF induction via LDL-receptor, and not scavenger receptors which are expressed in monocyte/macrophages rather than endothelial cells. Sakai *et al.* (1996) reported that oxidized LDL induces human monocyte-derived macrophage proliferation and that acetylated LDL alone does not. They concluded that both stimulation via scavenger receptors and cellular uptake of oxidized lipid, lysophosphatidylcholine, are needed for macrophage proliferation. The present results indicated that modified serum containing oxidized LDL was a more potent inducer of M-CSF production from monocytes than acetylated LDL, and that M-CSF induced by acetylated LDL was not sufficient for monocyte proliferation/survival. Furthermore, Sakai *et al.* (1997) demonstrated that protein kinase C is involved in the signal transduction triggered by oxidized LDL in murine macrophages, which may agree with the observation that PMA augmented M-CSF production from human monocytes. Therefore, it is possible that large amounts of M-CSF, induced by oxidized LDL, cause monocyte proliferation. To investigate the effect of oxidized LDL on M-CSF production and/or monocyte proliferation, further experiments with chemically and naturally oxidized LDL are in progress.

Scavenger receptor ligands such as dextran sulphate, poly(I) and heparin as well as acetylated LDL induced M-CSF production, indicating that stimulation via scavenger receptors leads to M-CSF production by monocytes. Goldstein *et al.* (1979) reported that heparin does not compete with acetylated LDL for binding to the receptors on murine macrophages. On the other hand, Falcone (1989) indicated a role of scavenger receptor in the induction of urokinase by heparin. The reason for the discrepancy between the two reports remains to be determined. However, the present results demonstrate that heparin as well as other scavenger receptor ligands induce M-CSF production in human monocytes.

Recent studies have demonstrated expression of M-CSF in atherosclerotic lesions using *in situ* hybridization and immunohistochemical staining, and have indicated that macrophage-derived foam cells prepared from atherosclerotic aortas produce M-CSF (Rosenfeld *et al.* 1992). On the other hand, suppressive effects of exogenously administered M-CSF on atherogenesis have been reported (Inoue *et al.* 1992). The present results showed that M-CSF production from monocyte/macrophage is induced by various scavenger receptor ligands, which might explain one of the mechanisms of M-CSF expression in the atherosclerotic intimal cells.

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