Human arterial wall cells secrete factors that are chemotactic for monocytes

(atherosclerosis/macrophages/arterial smooth muscle cells)

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Communicated by Earl P. Benditt, May 16, 1983

ABSTRACT Macrophages and arterial smooth muscle cells comprise the cellular components of the atherosclerotic plaque. The vessel wall accumulation of macrophages occurs by a process of increased circulating monocyte migration into the vessel wall. In these studies it is demonstrated that human macrophages and arterial smooth muscle cells in culture secrete potent chemotactic factors for freshly isolated human monocytes. In contrast, human fibroblast-conditioned medium has no chemotactic activity. The effect of macrophage-conditioned medium is a function of macrophage differentiation and can be potentiated by macrophage activation. These results suggest that secretory products of human macrophages and arterial smooth muscle cells may be important stimuli for increased monocyte migration into the vessel wall *in vivo*.

Atherosclerotic plaques are characterized by vascular wall collections of lipid-laden cells referred to as "foam cells." There is now substantial evidence that these cells originate from arterial smooth muscle cells and from monocyte-derived macrophages (1-5). Intimal accumulation of smooth muscle cells occurs as a result of cell division (5, 6), which may be induced by locally active mitogens such as those released by platelets (7), macrophages (8), or endothelial cells (9). Intimal accumulation of macrophages probably results from the migration of blood-borne monocytes into the developing plaque (10), but little is known of the factors that might induce the migration of monocytes into the vascular wall. We now report that conditioned media from cultured human macrophages and arterial smooth muscle cells are chemotactic for freshly isolated human monocytes. These results indicate that cells that are present in the nascent plaque can secrete factors that, if present *in vivo*, could mobilize monocytes into the vascular wall, thereby promoting growth of the lesion.

MATERIALS AND METHODS

Cell Culture. Human monocytes were prepared from human blood by counterflow centrifugation as described (11). These preparations are approximately 95% monocytes as demonstrated by nonspecific esterase stain, cell volume, or Fc receptor status (11). The monocytes were plated at 0.5×10^6 cells per 35-mm dish in RPMI 1640 medium containing 20% autologous serum and allowed to differentiate into macrophages. Human arterial smooth muscle cells were grown from microscopically normal intimal-medial explants of surgical specimens from human femoral artery (obtained from patients undergoing surgery for peripheral vascular disease) or from human aorta (obtained from children undergoing correction of coarctation) by previously described methods (12). Arterial smooth muscle cell cultures obtained in this manner have previously been shown

to yield a relatively pure population of smooth muscle cells by light and electron microscopy (12). Cultured human skin fibroblasts were grown from skin biopsies obtained from the anterior thighs of normal volunteers as described (13). Arterial smooth muscle cells and fibroblasts were grown in modified Dulbecco-Vogt medium containing 10% pooled human serum. Cells from the third or greater subculture were allowed to reach near confluence in 35-mm dishes before collection of conditioned media.

Prior to the collection of conditioned medium, arterial smooth muscle cells, macrophages, or fibroblasts were washed three times with RPMI 1640 medium and were then incubated in medium alone or in medium containing 0.2% bovine serum albumin. After a 24-hr incubation, media were collected, spun, and frozen at -20°C until used in the chemotaxis assay. Unless otherwise specified, macrophage-conditioned medium was collected from day 5-6 cultures and arterial smooth muscle cell- and fibroblast-conditioned media were collected when cells reached near confluence.

For experiments testing the effect of macrophage stimulation on the chemotactic activity of conditioned medium, human monocyte-derived macrophages were isolated and cultured in buffer and media made with pyrogen-free water. On day 6 of culture, the cells were washed three times with serum-free medium and then were incubated overnight with $1\ \mu\text{g}$ of bacterial lipopolysaccharide per ml. The following morning phorbol 12-myristate 13-acetate was added to a final concentration of $1\ \mu\text{g}/\text{ml}$. Two hours later the medium was collected for assay of its chemotactic activity. Control cultures were incubated for the same period in serum-free medium alone. Lipopolysaccharide and phorbol 12-myristate 13-acetate were added to the control conditioned media at concentrations equal to those in the stimulated culture media.

Chemotaxis Assay. Chemotaxis assays were performed in modified blind well chambers as previously described (14). Monocyte migration was measured by using a modification of the radiolabeled chemotaxis assay of Gallin *et al.* (15). Purified monocytes were prepared as previously described, except that calcium- and magnesium-free Krebs-Ringer phosphate buffer was used during cell isolation. At the end of the isolation procedure, the monocytes were taken up in Hanks' balanced salt solution and labeled with ^{51}Cr ($10\ \mu\text{Ci}$ per 10^6 monocytes; $1\ \text{Ci} = 3.7 \times 10^{10}\ \text{Bq}$) by incubation for 1 hr at 37°C with intermittent gentle agitation. The labeled cells were washed twice in Hanks' balanced salt solution and finally taken up in 2% bovine serum albumin/RPMI 1640 to a final cell concentration of 1.5×10^6 cells per ml. An aliquot was taken from the final cell suspension to determine the ratio of cell-associated to total ^{51}Cr radioactivity; this always exceeded 95%.

The conditioned medium was added to the lower well of the chemotaxis chamber and covered with a $3\text{-}\mu\text{m}$ cellulose nitrate filter, above which was placed a $5\text{-}\mu\text{m}$ polycarbonate filter. The

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labeled cells (1 ml) were added to the upper compartment and the chambers then were incubated for 90 min at 37°C in a humidified environment of 5% CO₂ in air. At the end of the incubation the chambers were disassembled and the cellulose nitrate (i.e., lower) filters were removed and assayed for ⁵¹Cr radioactivity. The numbers of cells that had migrated into the lower filter was calculated by dividing the cpm present in the lower filter by the cpm per monocyte. In all experiments, 0.2% bovine serum albumin/RPMI 1640 or RPMI 1640 alone, incubated in the absence of cells, served as the negative control.

RESULTS

Conditioned media from cultured human macrophages and arterial smooth muscle cells were chemotactic for freshly isolated monocytes in a dose-dependent fashion (Fig. 1 A and B). Checkerboard analyses were performed to differentiate true chemotaxis from chemokinesis (16) (Table 1). The stimulation of monocyte migration occurred along a positive concentration gradient of conditioned medium, thereby confirming the chemotactic nature of the conditioned media. In most experiments

FIG. 1. (A) Dose-response relationship between macrophage-conditioned medium and monocyte migration. Macrophage-conditioned medium (MCM) was collected from day 5–6 human macrophages in 0.2% bovine serum albumin/RPMI 1640. Before addition to the chemotaxis chamber, MCM was diluted to various concentrations with fresh 0.2% bovine serum albumin/RPMI 1640. The y-axis intercept represents the effect of 0.2% bovine serum albumin/RPMI 1640 that had been incubated in the absence of cells. The points represent the mean of duplicate (no standard error bars) or triplicate determinations. (B) Dose-response relationship between arterial smooth muscle cell-conditioned medium (ASMC-CM) and monocyte migration. Arterial smooth muscle cell-conditioned medium from nearly confluent human arterial smooth muscle cells was used as described in A. Points represent the mean $\pm$ SEM of triplicate determinations.

Table 1. Checkerboard analysis of macrophage- and arterial smooth muscle cell-conditioned media

MCM, no. of cells in lower filter $\times 10^{-4}$				ASMC-CM, no. of cells in lower filter $\times 10^{-4}$			
% (lower well)	% (upper well)			% (lower well)	% (upper well)		
0	0	37.5	75	0	0	37.5	75
0	7.2	8.6	6.5	0	9	4	6
37.5	16.9	8.7	6.0	37.5	16	9	6
75	16.4	9.8	5.1	75	25	11	7

Macrophage-conditioned medium (MCM) or arterial smooth muscle cell-conditioned medium (ASMC-CM) was placed in the upper or lower wells of chemotaxis chambers at varying concentrations. The labeled monocytes were always placed in the upper well. Those combinations produced positive or negative concentration gradients of conditioned medium between the cells and the lower filter. The numbers represent the mean of duplicate or triplicate determinations.

a standard chemoattractant as 3% endotoxin activated serum or N-formyl-methionyl-leucyl-phenylalanine (1 nM) was included as a positive control. The monocyte chemoattractant activity present in macrophage and arterial smooth muscle cell culture supernatants usually exceeded that present in the positive control (data not shown).

Macrophage-conditioned media for these experiments were collected from day 5–6 cultured macrophages. Because the pattern of secretory products from monocyte-derived macrophages may change as cell differentiation proceeds (17), the effect of time in culture on the chemotactic activity of monocyte-derived macrophage-conditioned medium was examined (Fig. 2). A low level of chemotactic activity was detected as early as 3 hr after the monocytes were placed in culture. The level of this activity remained stable for 2–3 days but increased significantly at day 4 and remained at this high level through day 10.

Macrophage activation has been related to a number of changes in macrophage metabolism and function *in vivo* and *in vitro* (17–19). The effect of macrophage stimulation on the chemotactic activity of conditioned medium was examined as shown in Fig. 3. Conditioned medium from stimulated cultures approximately doubled the number of monocytes (mean $\pm$ SEM) that migrated into the lower filter (22.4 ± 5.1 vs. $8.9 \pm 1.2 \times 10^4$ cells per filter).

Next the chemotactic activity of human arterial smooth mus-

FIG. 2. Chemotactic activity of macrophage-conditioned medium as a function of macrophage differentiation in culture. Macrophage-conditioned medium was collected in 0.2% bovine serum albumin/RPMI 1640 over 24 hr at the times shown. Points represent the mean $\pm$ SEM of triplicate determinations. The y-axis intercept represents the effect of 0.2% bovine serum albumin/RPMI 1640 alone.

FIG. 3. Effect of macrophage stimulation on the chemotactic activity of macrophage-conditioned medium. Macrophages were stimulated with bacterial lipopolysaccharide and phorbol 12-myristate 13-acetate, which were added to the unstimulated culture-conditioned medium prior to its use in the chemotaxis assay. Values represent the mean $\pm$ SEM of triplicate determinations.

cle cell- and human skin fibroblast-conditioned medium were compared over a range of cell densities. The chemotactic activity of arterial smooth muscle cell-conditioned medium was a positive function of cell density over the range of 15,000–60,000 cells per 35-mm dish. Human fibroblast-conditioned medium was not chemotactic at cell densities of 25,000–150,000 cells per 35-mm dish.

DISCUSSION

Monocyte-derived macrophages are an important component of developing arteriosclerotic plaques and may be the first cell type to accumulate in response to hyperlipidemic vessel wall injury (10, 20). These cells may be important in the progression of the atherosclerotic plaque for several reasons. They secrete a mitogen, the macrophage-derived growth factor, which induces arterial smooth muscle cell division, thereby contributing to local smooth muscle cell accumulation (5, 8). They also secrete factors that stimulate native low density lipoprotein uptake by arterial smooth muscle cells (21) and modified low density lipoprotein uptake by other macrophages (22). Both of these activities may contribute to the local formation of lipid-laden foam cells. Therefore, any factor that increases the local accumulation of macrophages in the vascular wall might be important in the pathogenesis of the plaque lesion. Jauchem *et al.* (23) recently have reported that conditioned medium from cultured baboon aortic smooth muscle cells is chemotactic for mononuclear cells, whereas conditioned medium from cultured baboon aortic endothelial cells is not. Platelet factor 4 (24) and platelet-derived growth factor (25) have also both recently been shown to be chemotactic for monocytes. If local platelet aggregation precedes macrophage accumulation, these platelet secretory products may be important in initiating monocyte migration into the vascular intima. However, recent evidence in hyperlipidemic animal models suggests that macrophage accumulation may precede the disruption of the endothelial cell layer and platelet aggregation in the genesis of the atherosclerotic plaque (2, 10, 20).

The present study demonstrates that both human macrophages and arterial smooth muscle cells secrete factors that are chemotactic for monocytes. That chemotactic activity is not a general feature of cultured cells is indicated by the observation that conditioned medium from fibroblasts, a cell type not thought to be important in the formation of the atherosclerotic plaque, is not chemotactic. This last result differs from that of Sobel and Gallin (26), who reported that fibroblast-conditioned medium was chemotactic for mononuclear cells. The reason for this difference is not clear but may relate to our use of only serum-free cell supernatants for assay, whereas serum-containing me-

FIG. 4. Comparison of conditioned medium from human fibroblasts and arterial smooth muscle in monocyte chemotaxis over a range of cell densities. Human fibroblasts and smooth muscle cells were plated over a range of densities and allowed to grow for 3 days. At that time conditioned medium was collected in 0.2% bovine serum albumin/RPMI 1640 over a 24-hr period. After collection of the conditioned media, the cells in each dish were trypsinized and counted. Values represent the mean $\pm$ SEM of triplicate determinations.

dium was employed in the earlier report.

The production of chemotactic activity by arterial smooth muscle cells must be constrained *in vivo* because normal vessel walls do not contain large numbers of macrophages. However, a locally occurring factor might allow secretion of this chemotactic factor to be initiated or increased. Conditions that might be important in stimulating production of arterial smooth muscle cell chemotactic factor must be investigated further. Our current data indicate that the phase of cell growth may not be important for the production of chemotactic activity by cultured human arterial smooth muscle cells. Chemoattractant activity was present in supernatants from cells that were presumably in logarithmic phase growth (Fig. 4) as well as in supernatants from cells that had been plated 7–10 days earlier (Fig. 1B) and had presumably reached stationary phase. However, this issue will require more rigorous examination before definitive conclusions can be made. The identity of these chemotactic substances will also require further investigation. Cultured arterial smooth muscle cells and macrophages have both been shown to secrete arachidonic acid metabolites (27, 28) and a number of proteins (28, 29) that are potential chemotactic agents (28, 30).

The observation that macrophage-conditioned medium contains a factor that is chemotactic for monocytes suggests that regardless of which factors might initiate monocyte migration into the vessel wall, macrophages, once present, can sustain directed monocyte migration and support the local accumulation of macrophages. Furthermore, if local activation of macrophages can be shown to occur in the vessel wall, the macrophage-induced migration of monocytes could be substantially potentiated.

The authors thank Carole Rainer for expert technical assistance and Sharon Kemp for skillful preparation of the manuscript. This work is supported in part by U.S. Public Health Service Grant AM 02456 and by a grant from R. J. Reynolds Industries. T.M. is a recipient of a National Institutes of Health Research Fellowship Award (HL 06131) and A.C. is a recipient of a Special Emphasis Research Career Award (AM 00592).

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