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Localized Adhesion of Monocytes to Human Atherosclerotic Plaques Demonstrated *in Vitro*

Implications for Atherogenesis

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Blood-derived macrophages in the arterial intima are a characteristic feature of active atherosclerotic plaques. Adherent monocytes on the luminal surface and increased adhesion molecules on the endothelium have suggested that specific molecular adhesion mechanisms are involved in monocyte/macrophage traffic into the arterial wall. Adhesion of human monocytes and related cell lines was therefore studied in vitro to histological sections of human plaques. At 37°C, these cells bound selectively to the plaques. Binding to the endothelium occurred and was also present extensively in the diseased intima. Inhibition studies showed that the endothelial and general intimal binding had largely similar molecular properties. Strong inhibition was produced by antibodies to the monocyte-specific adhesion molecule CD14, to β_2 integrins, and to ICAM-1. Likewise, a peptide containing the Arg-Gly-Asp sequence was strongly inhibitory, suggesting that binding of leukocyte integrins to arterial extracellular matrix was synergistic with cell-cell interactions. A P-selectin antibody was exceptional in giving selective inhibition of endothelial adhesion, which correlates with the specific endothelial localization of this adhesion molecule. These results show that monocytes adhere to atherosclerotic plaques through the focal activation of multiple arterial wall adhesion molecules, confirming the adhesion hypothesis. A positive feedback theory for the pathogenesis of atherosclerosis can be suggested, based on the ability of macrophages in the wall to activate the endothelium, induce adhesion molecules, and facilitate additional monocyte entry. The adhesion assay provides a means for the identification of adhesion inhibitors with therapeutic potential. (Am J Pathol 1996, 149:73–80)

Atherosclerosis is responsible for nearly one-half of deaths in the Western world and thus represents one of the major challenges facing medicine today. Research to date has failed to identify factors that determine accurately the risk of disease in an individual, suggesting that there are major mechanisms remaining undiscovered.

In atherosclerosis, it is well established that monocytes can be seen adhering focally to the arterial endothelium over the plaque lesions.[1–4] Recently, an animal model showed that the adhesion of leukocytes to arteries was enhanced by smoking, a well known risk factor for atherosclerosis.[5] After adherence, monocytes migrate across the endothelium and develop into large lipid-laden macrophages. These cells, together with proliferating endogenous smooth muscle cells, extracellular lipid, and cell matrix deposits, form an atherosclerotic plaque within the arterial intima, which thickens the wall and occludes the lumen. Growth factors from macrophages are likely to play a role in the cell proliferation.[6]

Adhesion, under flow conditions, of leukocytes in suspension to histological tissue sections was introduced by Stamper and Woodruff[7] to study lymphocyte traffic into lymph nodes. The specific adhesion observed to vessels was seminal to the concept of leukocyte-endothelial adhesion. Subsequently, it has been established that this adhesion is mediated by complementary adhesion molecules on the leukocytes and the endothelial cell and that adhesion so produced is an essential mechanism for the leuko-

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cyte traffic of inflammation.[8] Similar adhesion mechanisms have been implicated in atherosclerosis by the finding of focal increases in the adhesion molecule VCAM-1 in the arterial endothelium of plaques in the rabbit[9] and of ICAM -1, E-selectin,[10–12] and P-selectin[13] in the human. Monocyte adhesion can also be investigated by adhesion to tissue sections, as shown by the binding of U937 promonocyte cells to cerebral vessels in experimental allergic encephalomyelitis.[14] Monocytes make use of similar adhesion interactions as other leukocytes but also express a specific cell surface molecule with adhesive properties, CD14. CD14 is a 55-kd mucin-like cell surface molecule with dual function. It acts both as an endotoxin receptor[15] and as an adhesion molecule binding cytokine-activated endothelium.[16,17] CD14 when cross-linked allows the activation of β_2-integrin/ICAM-1-dependent adhesion[18–20]; so an initial CD14-mediated adhesion might be strengthened by secondary interactions. Here we report on the binding of human monocytes and monocyte cell lines to histological sections of human atherosclerotic plaques.

Materials and Methods

Monocytes were isolated from normal human peripheral blood by Lymphoprep density gradient centrifigation and isolated by adherence to plastic for 1 hour at 37°C, followed by treatment with phosphate-buffered saline (PBS)-EDTA for 5 minutes to facilitate release and then washing in medium. Purity of monocytes adherent in the assay was shown by the staining by peroxidase immunohistochemistry of cell smears and of the adherent cells after adherence on the artery sections with the macrophage antibody HAM56 (A. Gown), when > 90% were positive in each. The ability of monocytes to bind to atherosclerotic artery, even when not isolated by plastic adherence, was demonstrated by HAM56 positivity in a subset of the cells adherent to an artery after their separation from blood on a Lymphoprep gradient alone. However, the occurrence of activation during isolation by either technique cannot be excluded. U937 promonocyte-like and THP-1 macrophage-like cells (European Tissue Culture Collection, Porton Down, UK) were maintained in tissue culture, stimulated with 10 ng/ml phorbol myristyl acetate 24 hours previously as required, and harvested by agitation. All cells were washed in RPMI-1640 with 10% fetal calf serum and 10 mmol/L HEPES before the assay. Extensive preliminary experiments were done to optimize assay conditions. Cryostat sections were cut from atherosclerotic human carotid endarterectomy specimens from elderly patients onto silane-treated microscope slides[10] on the day of the assay, dried, and used unfixed. Postmortem arteries were used to study medial and adventitial adhesion. The lesions in the specimens had been characterized by immunohistochemistry[10]; fibro-fatty plaques and non-atherosclerotic areas with intact endothelium were used. The non-atherosclerotic areas usually showed diffuse intimal thickening but are referred to as normal in the figures, as the thickening is a near-physiological change in aged arteries. U937 and THP-1 experiments were normally done in triplicate, and blood monocytes were done with single or duplicate slides. A circle of approximately 12 mm diameter was drawn with a hydrophobic marker pen (Dako, Wycombe, UK) around the section, and 200 μl of cell suspension was added. Cells were used at between 10^6 and 1.5×10^7/ml, routinely at the higher figure, at which greater adhesion was produced. A 60-ml volume of blood provided sufficient monocytes for four slides. The slides were placed on a horizontally rotating table (Luckham R100 Rotatest, radius of gyration 1.8 cm) and rotated at 60 rpm for 40 minutes at 37°C. The slides were then dipped rapidly five times into PBS to remove non-adherent cells. They were then immersed in 3% paraformaldehyde in PBS for 15 minutes to fix the adherent cells and stained with hematoxylin. To confirm the integrity of the endothelium, some sections were stained by immunohistochemistry with a peroxidase-conjugated von Willebrand factor antibody (Dako) after the adhesion assay. Adhesion in atherosclerotic or normal areas was expressed as cells per ×400 microscope field for both endothelium and intima and was measured using a counting frame as cells/200 μm lengths of endothelium and as cells in 200 × 150 μm areas of the subendothelial intima. More than 10 ×400 magnification fields were counted for each observation on an artery, and results are given ± SEs of the fields. Adherent cells could be distinguished from endogenous cells by optical plane and morphology; the Olympus BH2 microscope used routinely gave better results than other microscopes for distinguishing adherent cells in a different optical plane. Cells within the fields were counted as binding to the endothelium if overlying it or if touching the luminal surface. The significance values of the differences between binding to atherosclerotic and normal areas were calculated by the Mann-Whitney *U* test.

For inhibition of adhesion, the following substances were used at the concentrations stated. Peptide Gly-Arg-Gly-Asp-Ser, 250 μg/ml; peptide Ser-Asp-Gly-Arg-Gly, 250 to 800 μg/ml. The following mouse monoclo-

nal antibodies were used at 20 or 25 μg/ml: CD18/β_2 integrin chain, MHM23 (Dako); ICAM-1, 8.4A6 (D. Haskard); VCAM-1, 4B9 (J. Harlan); P-selectin, LYP20 (J. McGregor); CD14 antibodies UCHM1 (Sigma, Poole, UK) and LeuM3 (Becton Dickinson, San Jose, CA); HLA-DR, YD1/63 (Seralab, Crawley Down, UK); CD29/β_1 integrin chain, 4B4 (Coulter Corp., Hialeah, FL). Other inhibitors were 100 μg/ml wheat germ agglutinin (WGA; Sigma) and WGA with 100 mmol/L *N*-acetyl glucosamine (NAG; Sigma). MOPC21 and UPC10, mouse plasmacytoma proteins of IgG_1 and IgG_{2a} subclasses (Sigma), were used as controls at 20 μg/ml.

Statistical analysis of numbers of cells bound in duplicate or triplicate inhibition experiments was on predetermined arbitrary pairs of test and control slides. Two-way analysis of variance was calculated on log-transformed data from the arterial field measurements, using macro software based on the general linear model test, Minitab 7.1 (Minitab, Inc., State College, PA), with the factors being test/control status and pair grouping. If the general linear model statistic was invalidated by demonstration of significant interactions between the factors, then the Mann-Whitney *U* test was applied between the same set of pairs, and the least significant value in the set was taken as the value for the experiment. A *P* value < 0.05 was considered significant.

The binding of antibodies to U937 cells was assayed by immunohistochemistry on acetone-fixed cell smears and quantitated by hue/saturation/intensity color image analysis in 10 high power fields as described,[22] using intensity thresholding to detect the cell images, with the results expressed as mean percentage area of cell images stained.

Limulus lysate assays for endotoxin (Sigma) were done according to the manufacturer's instructions.

Results

At 4°C, under similar conditions to previous studies,[7] no adhesion to arterial sections occurred. However, at 20 or 37°C, human peripheral blood monocytes and U937 cells showed selective binding to atherosclerotic plaques, compared with non-atherosclerotic arterial intima (Figure 1). THP-1 cells also gave a highly selective binding to plaques (not shown). Specificity of binding to monocyte-like cells was demonstrated by negligible adhesion with a hybridoma cell line. U937 adhesion was markedly enhanced after stimulation of the cells with phorbol myristyl acetate.[23] This paralleled an increase in expression of CD14 and major histocompatibility class

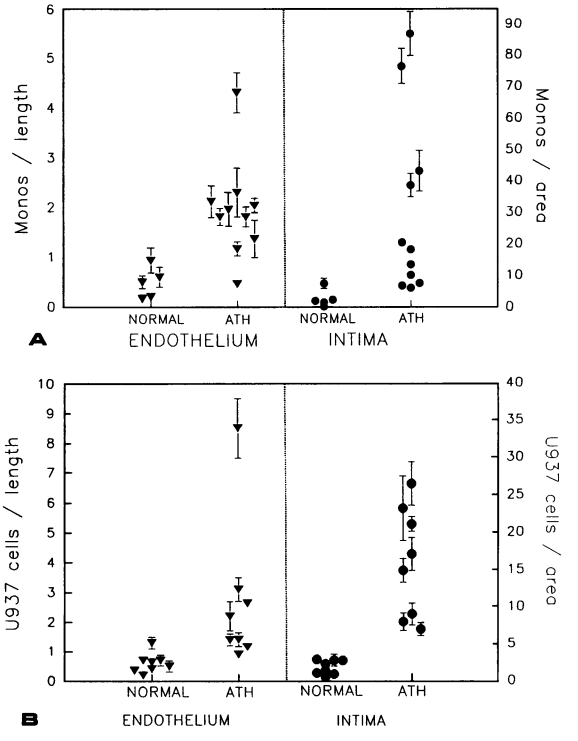

Figure 1. *Binding of blood monocytes and U937 cells to atherosclerotic and normal intima.* **A**: *Human blood monocytes. The differences between atherosclerotic and normal areas are significant* ($P < 0.007$) *in both endothelium and intima.* **B**: *Stimulated U937 cells. The differences are significant* ($P < 0.002$) *in both endothelium and intima. Cells in* **A** *and* **B** *are both* 1.5×10^7*/ml.*

II (see below). Adhesion occurred to the endothelium of the plaque area (Figure 2A), with some of the cells attaching and flattening on the luminal surface of the endothelium, strikingly similar to the binding of monocytes to plaques *in vivo*.[1–3] The binding to non-atherosclerotic artery endothelium was much less, and the difference was highly significant (Figure 1). Endothelial specificity of the adhesion was confirmed by staining of the endothelial cells for von Willebrand factor (Figure 2A). Unexpectedly, all of the types of cells tested also adhered extensively to the atherosclerotic intima outside the endothelium (this is referred to as intimal adhesion), whereas adhesion to non-atherosclerotic intima was much less, and the difference was highly significant (Figures 1 and 2). When adhesion was strong, the adherent cells formed tight clumps, suggesting that monocyte- monocyte adhesion was induced *in situ* (Figure 2C). Areas of the intima showing adhesion were usually cellular, containing either smooth muscle cells or macrophages. Variable levels of adhesion were also seen in the media, adventitia, and extra-arterial connective tissue, but apart from some increase in medial adhesion, they did not relate to atherosclerotic plaques. U937 adhesion to rabbit ath-

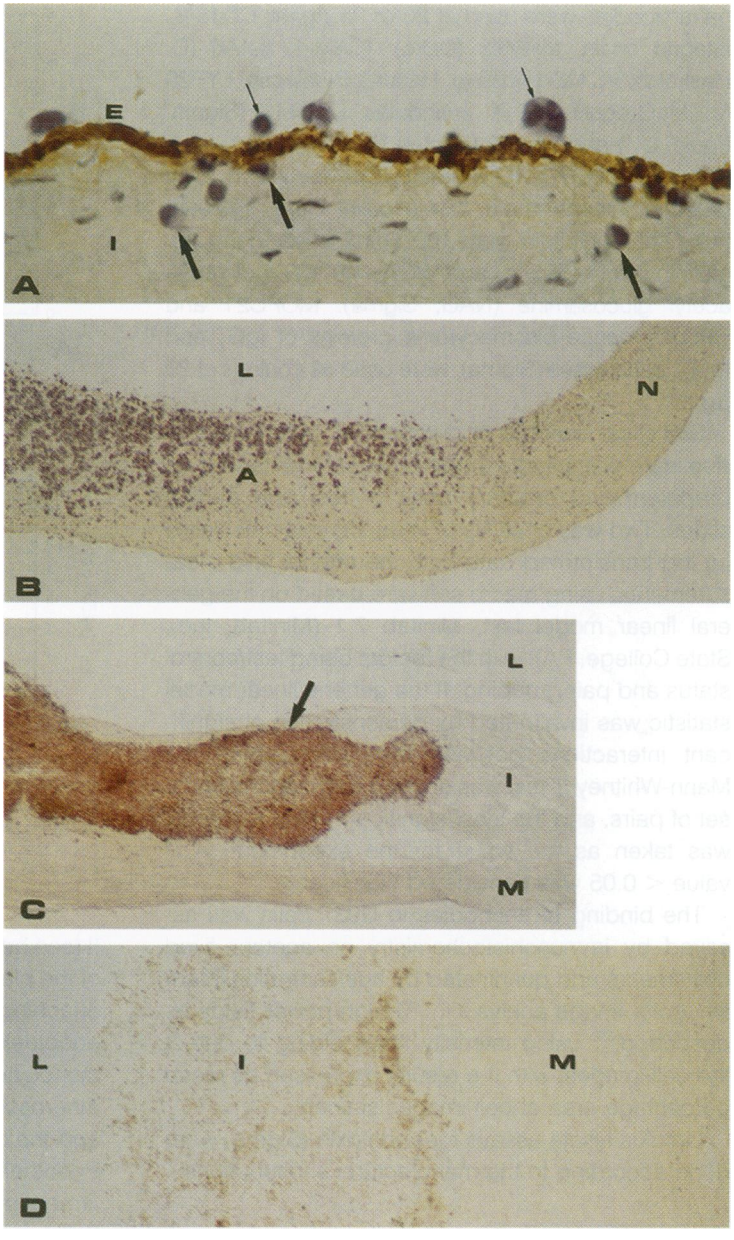

Figure 2. *Histology of adhesion reactions to atherosclerotic arteries.* A: *Binding of U937 cells to the inner part of a fibro-fatty plaque. The endothelium (E) is stained brown by immunohistochemistry for von Willebrand factor.* Thick arrows *indicate U937 cells binding to the endothelium and sub-endothelial intima.* Thin arrows *indicate U937 cells touching and flattening on the luminal border of the endothelium. Original magnification, ×630. The area shown is slightly smaller than those counted for quantitative studies.* B: *Low power (original ×25) magnification of fibro-fatty plaque-related intimal adhesion of U937 cells. Almost all of the purple hematoxylin staining is due to U937 cells. They are adherent to the atherosclerotic intima (A); the thinner normal part of the arterial wall (N) shows minimal adhesion. This specimen shows a level of adhesion typical of the study.* C: *Low power view (×25) of U937 fibro-fatty plaque-specific intimal adhesion of unusually high density, forming a discrete mass of hematoxylin-stained adherent cells* (arrow). *Arterial wall cells are contributing little to the staining seen.* D: *Cross-section of an artery (same specimen as in* C*) with blood monocytes adherent specifically at high density to atherosclerotic intima. L, lumen; I, intima; M, media. The monocytes are stained brown by immunohistochemistry with HAM56 anti-monocyte/macrophage antibody, and arterial wall cells are seen stained very lightly with hematoxylin. Original magnification, ×100.*

erosclerotic artery was much less than to human lesions, demonstrating species specificity. Addition of EGTA to the assay resulted in near-total abolition of all adhesion, showing divalent cation dependence.

With U937 cells, studies were done with antibodies of known inhibitory capacity to identify the adhesion molecules involved. A β_2 integrin chain (CD18) antibody gave consistent inhibition (Figure 3A) of endothelial and intimal adhesion, as did an ICAM-1 antibody. Comparison of the adhesion reactions with ICAM-1-stained sections cut serially showed strong adhesion in ICAM-1-positive areas of intima. By contrast, a VCAM-1 antibody was without inhibitory effect (Figure 3A).

An Arg-Gly-Asp sequence-containing peptide gave marked inhibition (Figure 3A) that was dose-related, whereas a control peptide had no effect (Figure 3A). The Arg-Gly-Asp sequence is found in integrin ligands that are widespread interstitial matrix proteins, for example, fibronectin, vitronectin, fibrinogen, and von Willebrand factor.[24] Several bind to the β_1 family of integrins; however, an antibody, 4B4, to the β_1 integrin chain gave variable, often weak inhibition (not shown), suggesting a limited role for that molecular component.

An antibody to P-selectin gave selective inhibition of endothelial adhesion (Figure 3A) without affecting binding to the bulk of the intima. This result corre-

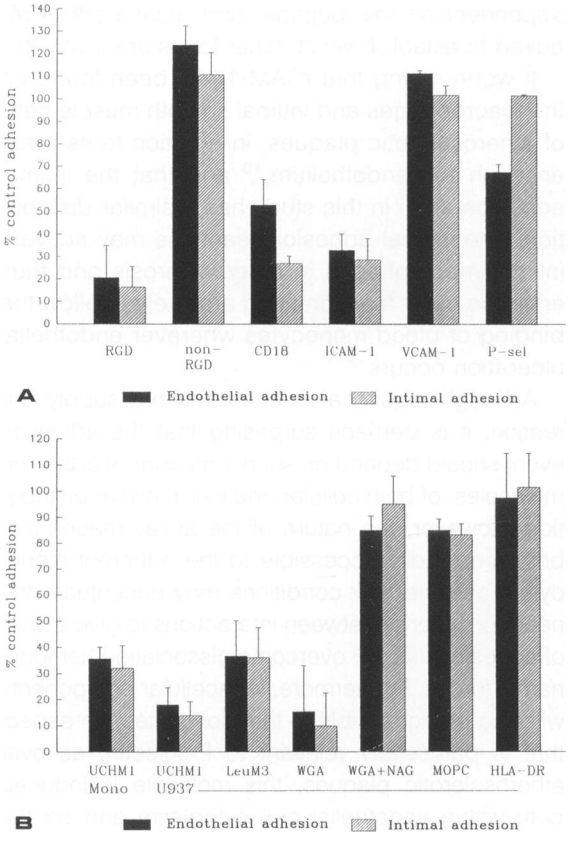

Figure 3. *Adhesion blockade by inhibitors. Results are expressed as percentage of control adhesion.* A: *Effect of blocking agents against integrin, Ig superfamily, and selectin adhesion molecules on stimulated U937 cell adhesion to atherosclerotic arteries. The means ± SEs of three or more triplicate experiments are shown, except P-selectin, which was two experiments. RGD, Arg-Gly-Asp peptide; non-RGD, control peptide. The remaining bars show inhibition by antibodies to the molecules indicated. Statistical significance of inhibition applies in all replicates of experiments unless otherwise stated: RGD/intima,* $P < 0.004$*; RGD/endothelium,* $P < 0.05$ *in two of three experiments; non-RGD, not significant; CD18 and ICAM-1,* $P < 0.02$ *to* 0.001*; VCAM-1, not significant; P-selectin/endothelium,* $P < 0.001$. B: *Effect of CD14 antibodies, related blocking agents, and controls on blood monocyte and stimulated U937 cell adhesion to atherosclerotic arteries. Means ± SEs of three or more experiments, except WGA + NAG, which is the result of one assay showing means and SEs of triplicate slides. UCHM1/Mono, inhibition of blood monocyte adhesion by UCHM1 anti-CD14 antibody (duplicate slides); the remaining data show U937 adhesion in triplicate slide experiments. LeuM3, anti CD14; WGA, wheat germ agglutinin; WGA + NAG, WGA with* N*-acetyl glucosamine; MOPC, MOPC21 control IgG; HLA-DR, antibody YD1/63. Inhibition was significant* ($P < 0.008$ *to* 0.001) *in all experiments with UCHM1, LeuM3, and WGA. Inhibition with other agents was not significant except one value of 77% of control* ($P < 0.02$) *with endothelium in one of three MOPC21 experiments.*

lates with the selective expression of P-selectin on endothelium and shows that endothelial adhesion could be identified specifically in the assay, even when it was adjacent to extensive adhesion to other intimal components. L-selectin, a selectin adhesion molecule expressed on leukocytes, was not investigated, as no expression was detected on U937 cells.

With the arteries, two antibodies to CD14, Leu-M3 (IgG_{2b}) and UCHM1 (IgG_{2a}), gave marked inhibition of adhesion. Inhibition was seen with both U937 cells and blood monocytes as targets (Figure 3B). Specificity of inhibition in the antibody experiments was shown by lack of inhibition of U937 cell adhesion with antibodies to HLA-DR, to CD68 (a monocyte intracellular antigen), or with the mouse non-immune immunoglobulins MOPC21 (IgG_1) and UPC10 (IgG_{2a}) (Figure 3B and observations not shown). HLA-DR is well expressed on stimulated U937 cells. Image analysis on peroxidase-stained cells showed that CD14 and HLA-DR antibodies gave 34 and 30%, respectively, of cell area stained with 24-hour phorbol myristyl acetate stimulation, and 36 and 48% at 48 hours, demonstrating that these antibodies were binding at similar levels. Nonstimulated cells gave less than 3% staining with both antibodies. The failure of inhibition of adhesion with the HLA-DR antibody thus indicates that an antibody binding to a major cell surface protein does not give rise to inhibition purely through a nonspecific steric hindrance mechanism. As CD14 can also function as a receptor for complexed endotoxin, mediating monocyte activation,[15] *Limulus* tests for endotoxin on the complete tissue culture medium were performed; they were consistently negative.

Adhesion was abolished by the NAG- and CD14-reactive lectin, WGA (100 mg/ml). However, there was no inhibition when NAG was added together with WGA (Figure 3B). These results parallel previous data on monocyte adhesion to cytokine-activated endothelium *in vitro*,[25] and WGA may inhibit adhesion through its ability to bind CD14.[17]

Discussion

These studies have confirmed the existence of major monocyte adhesion events in atherosclerosis, and it is highly probable that the endothelial adhesion has an important role in the recruitment of macrophages to atherosclerotic plaques. The findings that binding occurs to mesenchymal cells in the intima and that interstitial matrix molecules can be implicated in both endothelial and intimal adhesion allow novel extensions to the concept of monocyte-tissue adhesion in atherosclerosis. Furthermore, chemoattractant factors for monocytes have also been detected in the plaque endothelium,[26] and these are synergistic with adhesion molecules in cell migration.[8] As macrophage accumulation is a characteristic and specific feature of the atherosclerotic plaque,[1–3] it is likely that these adhesion events play a central role in its pathogenesis and may be rate-limiting factors in the process. Lesser numbers of T lymphocytes also ar-

rive, probably by similar mechanisms, and may contribute to events in the plaque.

Recent studies give good evidence for oxidized low density lipoprotein (ox-LDL) being involved in adhesion. Material with the physical and immunological properties of ox-LDL has been detected in atherosclerotic plaques.[27] Endothelium treated with ox-LDL shows specific adhesion of monocytes compared with other leukocytes[28] and synthesizes cytokines that induce monocyte chemotaxis and activation.[29] It is important to note that macrophages are highly potent in oxidizing LDL,[30] strongly suggesting that they do this in the plaque, although other cells present may also contribute.

The previously unrecognized involvement of the monocyte-specific molecule CD14 in atherosclerosis provides a mechanism for monocyte-specific adhesion and recruitment into the plaque. CD14 has previously been identified as a monocyte-specific adhesion molecule, but its tissue ligand has not been identified.[16,17] From the CD14 dependence of both endothelial and intimal adhesion, the ligand could be another inducible adhesion molecule that appears at both sites. A novel adhesion molecule recognized by the monoclonal antibody IG9 has these characteristics and may play a part in ox-LDL-induced endothelial/monocyte adhesion.[31]

An alternative view on the function of CD14 is that it is an important membrane-bound transducer of cell activation, with its own adhesion properties possibly having a triggering role. The previous evidence of CD14-dependent β_2 integrin activation[18–20] raises the possibility that monocyte β_2 integrin to ICAM-1 binding could follow the engagement of monocyte CD14 with a tissue ligand or other activator. The observed temperature dependence of monocyte-plaque adhesion could result from the metabolic requirements of intracellular activation events. The effective inhibition of monocyte-plaque adhesion by CD14, ICAM-1, or β_2 integrin antibodies even when each is applied separately is also compatible with a CD14-integrin activation sequence. The monocyte clumping observed after adhesion to plaque intima (Figure 2C) likewise could be mediated by homotypic β_2 integrin-ICAM-1 binding subsequent to cellular activation.

The inhibition of adhesion with an Arg-Gly-Asp peptide was found with both endothelial and intimal sites and thus suggests a role for monocyte integrin-extracellular matrix protein interactions in each. The integrin ligands fibronectin and von Willebrand factor are synthesized and released by endothelial cells[32] and are also present within the intima. The integrins involved will not be of the β_2 family, as they are not dependent on this peptide, and more work is required to establish which other forms are involved.

It worth noting that ICAM-1 has been found on the macrophages and intimal smooth muscle cells of atherosclerotic plaques, in addition to its presence on the endothelium,[10] and that the intimal adhesion seen in this study has a similar distribution. The intimal adhesion reactions may activate intimal macrophages in atherosclerosis and thus enhance other functions and are likely to allow the binding of blood monocytes wherever endothelial ulceration occurs.

Although sequential mechanisms may supply one reason, it is perhaps surprising that the adhesion event should depend on such a number of adhesion molecules, of both cellular and extracellular distribution. However, the nature of the assay means that both are readily accessible to the adherent monocytes, and the flow conditions may accentuate the need for synergy between interactions to give a level of adhesion that will overcome dissociating hemodynamic forces. Furthermore, intracellular components will also be accessible to the monocytes, an aspect that is particularly relevant to P-selectin, as over atherosclerotic plaques, this molecule is induced both within endothelial cell cytoplasm and on the luminal surface.[13] Clearly, adhesion events *in vivo* will not be identical.

The adhesion assay described is useful, as it provides a means of modeling the adhesion events of atherosclerosis *in vitro*. It can produce data on the human disease and allows comparison with animal models. Species differences may exist; in particular, these results correlate with previous immunohistochemical studies that have indicated that VCAM-1 is not expressed in the atherosclerotic arterial endothelium in elderly human subjects[12] (extensive unpublished observations), unlike in the rabbit model. Furthermore, as it can be argued that monocyte adhesion may be a controlling factor in disease progression, it may also be a suitable target for therapy against atherosclerosis. The adhesion assay will be helpful in the identification of inhibitors of adhesion that could have therapeutic potential.

The mechanisms described can be integrated into a comprehensive hypothesis for atherogenesis. It is proposed that monocyte/macrophage recruitment may lead to a self-perpetuating macrophage-dependent focal chronic inflammatory process in the artery wall.[10] The process could commence with trivial endothelial stimuli. For example, hypercholesterolemia and hemodynamic stress can activate endothelial cells, inducing adhesion molecules. Once the macrophages are recruited, additional macrophage-dependent events will

follow, including endothelial activation. Macrophage-driven oxidation of LDL is most probably involved, but atheroma macrophages also produce tumor necrosis factor-α and other endothelium-activating cytokines.[33] In addition, atheroma macrophages produce growth factors that are likely to induce smooth muscle proliferation[6] (unpublished observations). As a result, the lesion can be anticipated to develop in a focal fashion from the site of the initial insult, giving rise to the characteristic plaque morphology. Such a cyclical positive feedback mechanism is an ideal explanation for the locally random distribution of plaques usually observed, as it will amplify initial events and so allow an all-or-none response from the arterial wall. The absence of adhesion molecules in atypical advanced fibrous plaques lacking macrophages[10,13] fits well with the hypothesis, and it seems probable that they have lapsed into inactivity through loss of these cells. Genetic variation in the activity of the cycle components involved or environmental effects on them (eg, smoking on monocyte adhesion[5]) may contribute to the large component of individual risk of disease that is not so far understood.

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