Inhibition of Leukocyte Extravasation With a Monoclonal Antibody to CD18 During Formation of Experimental Intimal Thickening in Rabbit Carotid Arteries

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In the rabbit model of electrically induced intimal thickening, the adherence processes of different leukocyte subsets as well as the functional significance of leukocyte invasion in the initial migration of smooth muscle cells (SMCs) into the intima were studied by using monoclonal antibody (MAb) 60.3 (directed to the leukocyte adherence glycoprotein CD18), a known potent inhibitor of leukocyte adhesive functions. In control carotid arteries exposed to two periods of electrical stimulation within 36 hours, leukocytes, including all granulocyte subsets, monocytes, and lymphocytes, invaded the cell-free subendothelium. Concomitantly, SMCs were observed to migrate from the media into the intima. In the MAb 60.3-treated rabbits, however, neutrophil emigration into the stimulated arteries was abolished, whereas mononuclear leukocyte accumulation in the intima was only partially inhibited, indicating a complete CD18-dependent mechanism for neutrophil extravasation and additional receptor–ligand systems for the emigration of mononuclear leukocytes. SMCs moved into the intima despite complete blockage of neutrophils and the reduced accumulation of mononuclear cells within the subendothelium after MAb administration. These results preclude neutrophils as initiators of SMC migration into the intima. The influence of mononuclear cells on the migratory behavior of SMCs in intimal thickening formation, however, needs further elucidation. (Arteriosclerosis and Thrombosis 1992;12:997-1007)

KEY WORDS • intimal thickening • endothelium • leukocyte extravasation • leukocyte adhesion molecules • CD18 complex • smooth muscle cell migration • rabbit carotid artery

Adherence of monocytes and lymphocytes to the arterial endothelium, their transendothelial migration, and their accumulation within the intima have been frequently described as early events in human and experimental atherosclerosis.1-14 In advanced lesions also, significant amounts of mononuclear cells have been identified.6,15-20 Evidence exists that the mononuclear leukocytes, in addition to the well-recognized lipid-scavenging function of monocytes/macrophages, may contribute to lesion initiation and progression by secreting a variety of inflammatory mediators, cytokines, and growth factors (reviewed in References 21–24). Granulocytic cells, although attracting minor attention, have also been reported to occur in both human atherosclerotic plaques and experimentally induced intimal thickenings.6,13,16,17,25 Their functional significance in the development of intimal lesions is still a matter of speculation.25-27

In the model that induces intimal thickening in rabbit carotid arteries in response to electrical stimulation (ES),28 we previously demonstrated a massive initial invasion of both granulocytes and mononuclear cells into the developing lesion. This event was observed in the presence of a continuous yet functionally and structurally altered endothelium, and it preceded the accumulation of smooth muscle cells (SMCs) within the arterial intima.29,30 Within 4 weeks, intimal thickenings, primarily consisting of SMCs and to a minor extent mononuclear cells, developed in normocholesterolemic rabbits.29 These thickenings resembled fibromuscular intimal lesions formed either after balloon catheter injury31 or by perivascular manipulation.13,32-34 Initial events like endothelial damage and leukocyte involvement, however, differed between the various models. In addition, a cholesterol-rich diet fed during ES resulted in atheroma-type lesions containing macrophage-derived foam cells as well as lipid-laden SMCs and showing signs of necrosis,28,35 hence bearing some of the features of human atherosclerosis.

In the present study, interest was focused on 1) the processes mediating leukocyte adhesion and emigration into the vessel wall in this animal model and 2) the functional relation that might exist between leukocyte invasion and SMC migration into the arterial intima. Our tool in elucidating these problems was the monoclonal antibody (MAb) 60.3, which recognizes the leukocyte membrane glycoprotein CD18 and which has proven to be a potent inhibitor of leukocyte adherence...
Monoclonal Antibody

MAb 60.3 is a murine immunoglobulin (Ig) G\textsubscript{\textalpha} antibody that recognizes all three heterodimers of the membrane glycoprotein complex CD11/CD18 expressed on human leukocytes (B and T lymphocytes, monocytes, and polymorphonuclear leukocytes but not red blood cells, platelets, or cultured endothelial cells\textsuperscript{36}). It binds to a functional epitope on the common \beta\textsubscript{2}-chain (CD18) and has the advantage of cross-reactivity with cell-adhesion molecules of leukocytes from other species (reviewed in Reference 38). MAb 60.3 was prepared according to Beatty et al.\textsuperscript{39} Briefly, a BALB/c mouse was immunized with alloantigen-activated T cells. Ascites fluids were obtained from pristine primed BALB/c mice inoculated intraperitoneally with 5 x 10\textsuperscript{6} hybridoma cells. The antibody was purified by solid-phase absorption on staphylococcal protein A columns. The antibody concentrations in the stock solutions were 2-5 mg/ml. In cases where 0.01% NaN\textsubscript{3} was added to the antibody solutions, it was removed by exhaustive dialysis against sterile phosphate-buffered saline.

Animals and Experimental Design

Male New Zealand White rabbits (1.4–1.8 kg body weight, \(n=12\)), obtained from Thomae GmbH (Biberach, FRG), were used for the study. They were fed a normal rabbit standard diet (Altromin GmbH, Lage, FRG) during acclimatization in the animal department for at least 1 week as well as during the experiment. The right carotid arteries of the rabbits were exposed to transmural ES, which is known to elicit intimal thickening.\textsuperscript{28} In addition, one group of animals (\(n=7\)) received MAb 60.3 before as well as during ES; the others (\(n=5\)) received sterile saline as a control. Under anesthesia (8 mg metomidate hydrochloride and 0.07 mg fentanyl base/kg body wt), the MAb or saline injections were repeated twice at intervals of 12 hours, i.e., the second and third occurred 2 hours before the respective period of ES (Figure 1). Blood samples were drawn immediately before each MAb or saline administration, as well as at the end of the experiments for determination of total and differential leukocyte counts. Thirty-six hours after surgery, animals were anesthetized and their carotid arteries were perfused through the left ventricle at a pressure of 80–100 mm Hg with 0.1 M cacodylate-buffered 1.25% glutaraldehyde, as already described.\textsuperscript{26} The carotid arteries were carefully excised, the cuff was removed after earmarking the adventitial side next to the anode, and the vessels were stored in fixative (same composition as above).

The experimental protocol was reviewed and approved by the University of Tübingen Animal Care Committee.

Tissue Processing for Electron Microscopy

After immersion-fixation for at least 24 hours, the arteries were processed for transmission electron microscopy. The cuff-bearing artery segments were bisected, allowing examination of the vessel wall changes in the mid-region of the cylindrical segment. The left carotid arteries were also subdivided into rings approximately 3 mm long. All samples were postfixed in 1% OsO\textsubscript{4}, dehydrated through graded alcohols, stained en bloc in alcoholic uranyl acetate, embedded in Araldite, and sectioned.
and transversely cut into both semithin and ultrathin sections. Semithin sections were stained with toluidine blue and were used for light microscopic examination. Ultrathin sections were taken from the anodal area of the bloc face as revealed by light microscopy, stained with lead citrate, and examined with a Zeiss electron microscope 10.

Identification of Intimal Cells, Quantification, and Statistical Analysis

The composition of the intimal cell population of all specimens was analyzed with respect to the different cell types, which were identified on the basis of ultrastructural criteria. The subclasses of granulocytes, i.e., neutrophils, eosinophils, and basophils, were clearly identifiable by the distinctive, fine structural features of their specific granules as mentioned by Wetzel et al.41 Monocytes and lymphocytes were easily distinguished from granulocytes because of the absence of specific granules in their cytoplasm. Typical lymphocytes differed from monocytes because their nuclei were usually oval and the narrow rim of cytoplasm contained a tiny Golgi area, a small number of lysosomes, and few large mitochondria.42 However, sharp discrimination between the two cell types was not always possible, depending on the plane of the section. Thus, for a quantitative analysis of the intimal cell population, they were grouped as mononuclear cells. SMCs, although expressing different phenotypes, differed from intimal leukocytes in a variety of features including 1) thin basal lamina, which were sometimes only present in fragments; 2) abundant microcynocytic vesicles; 3) cytoplasm more or less packed with myofilaments; and 4) dense attachments on the cytoplasmic aspect of the membrane. The proportion of intimal cells not identifiable by these features or by those depicted in the figures amounted to, on average, 1±2%; this population consisted exclusively of cell profiles without a sectioned part of the nucleus.

Quantification was performed with ultrathin sections obtained from four different cutting planes per cuff-bearing segment that were at least 0.1 mm apart. The numbers of intimal cells that were arranged in one or two cell layers beneath the endothelium were determined for each cell type and expressed in relation to the number of endothelial cells (ECs) overlying the lesion in the anodal part. The data obtained from one animal were averaged, and the numbers of subendothelial cell profiles (with and without nuclei) belonging to a particular cell type were evaluated per 100 cross-sectioned ECs. The average values were used in the statistical analysis. The total count of ECs ranged from 416 to 522 per animal. For obtaining information about the relative cellular composition, the percentages of the different cell types within the intimal cell population were calculated.

All values reported represent mean±SD. The data on the composition of the intimal cell population were analyzed with the Wilcoxon test by comparing treatment and control groups.

To evaluate the effect of MAb treatment on total leukocyte counts in the circulating blood, leukocyte numbers were subjected to quadratic analysis of covariance. The target variate (leukocyte count) was incorporated as a gamma-distributed variable, time, and the square of this value represented the continuous explanatory variate.

Differential leukocyte counts in the peripheral blood were evaluated by a multiway analysis of variance. Global effects of both MAb treatment and time were tested in interaction with the different leukocyte subpopulations (model: cell type x treatment and cell type x time) after the percentage data were normalized with the arcsine transformation. Group means were compared with the Gauss test. Quadratic analysis of covariance, analysis of variance, and the Gauss test were performed by fitting generalized linear models from the software package GLIM 3.77,43 In all tests performed, significance was attributed to p<0.05.

Results

Leukocyte Invasion Into the Intima

In the electrically unstimulated left carotid arteries of both the MAb-treated rabbits and the control rabbits injected with saline, an intact endothelial lining without adhering blood cells covered an acellular subendothelial space. In the right carotid arteries of the control rabbits, however, exposure to two sessions of ES elicited structural changes in the endothelium, along with adherence and diapedesis of leukocytes, preferentially in the anodal part of the vessel wall. The ECs formed a continuous covering with normal-appearing junctional complexes, but individual cells exhibited ultrastructural features of perturbations, e.g., electron-dense cytoplasmic matrix, ruffled luminal surfaces, and dilated endoplasmic reticulum. The population of leukocytes adhering to and migrating through the endothelium consisted of representatives of all leukocyte subsets. Thirty-six hours after implantation of the electrodes, PMNs and monocytes predominated in the frequency of occurrence (Figures 2A and 2B). However, eosinophils and basophils as well as lymphocytes were occasionally encountered in association with the endothelium (Figures 2C and 2D). The observed stages of diapedesis indicated that the leukocytes preferentially used the intercellular pathway through junctional complexes for extravasation. At the examined time point, the different types of leukocytes were found not only adherent to and migrating through the endothelium but also lying within the subendothelium, where they were arranged in one or two layers of contiguous cells (Figure 3A). Some of the subendothelially located monocytes exhibited signs of transformation into macrophages (e.g., increased size, prominent Golgi complexes, and numerous lysosomes and mitochondria, as well as conspicuous endoplasmic reticulum). Single monocytes/macrophages and PMNs displayed protrusions toward the internal elastic lamina (Figure 3B), passed through it (Figure 3C) or were even found within the luminal part of the media. The outer media, however, was devoid of granulocytic and mononuclear cells at this time; together with the observations of transendothelial migration, this indicates that the leukocytes invaded the intima from the luminal side. In addition to the aforementioned types of inflammatory cells, plasma cells were occasionally found within the intima (Figure 3D).

Quantitative analysis of the intimal cell population in the control animals revealed that leukocytes were present in the subendothelium an average of 23±11 per 100 overlying, cross-sectioned ECs. Of these resident intimal leukocytes, PMNs constituted 21±12%, basophils 0.3±0.6%, eosinophils 0.2±0.4%, mononuclear cells 77±14%, and plasma cells 2±3%.
FIGURE 2. Photomicrographs of leukocytes associated with the endothelium of electrically stimulated carotid arteries from rabbits not treated with MAb 60.3. Panel A: Polymorphonuclear neutrophil (PMN) sending several protrusions into interendothelial gaps. Note the close membrane apposition between the neutrophil and the endothelial cells (E). Panel B: Monocyte (M) protruding through the endothelium (E) to the subendothelial space (SES). IEL, internal elastic lamina. Panel C: Lymphocyte (L) characterized by an oval nucleus with deep indentations and a narrow rim of clear cytoplasm is attached to the endothelium (E). Panel D: Eosinophilic granulocyte (EO) containing crystalloid structures (arrows) within its granules is lying between two endothelial cells (E). Bars = 1 μm.

In the MAb-treated rabbits, the accumulation of leukocytes in the intima next to the anode of carotid arteries exposed to two periods of ES was clearly reduced. As shown in Figure 4, PMNs were totally absent in the intima of MAb-receiving animals, whereas basophil and eosinophil counts remained unchanged.
FIGURE 3. Photomicrographs of leukocytes accumulating within the subendothelium from control rabbit carotid arteries after exposure to the stimulation program for 36 hours. Panel A: Polymorphonuclear neutrophil (PMN) and monocytes/macrophages (MΦ) are arranged in a single layer between the intact endothelium (E) and the internal elastic lamina (IEL). Panel B: Villous cytoplasmic processes (arrows) of a macrophage are protruding toward the internal elastic lamina. Note fragmentation of the lamina and a pseudopod of a smooth muscle cell (arrowhead) next to the protrusions. Panel C: Macrophage squeezing through the internal elastic lamina. E, endothelium. Panel D: Plasma cell (P) with its distinctive well-packed, rough endoplasmic reticulum lying beneath the endothelium and making contact with a macrophage. Bars=2 μm.
Degenerating granulocytes were rarely seen attached to the endothelium, but they were never found within the subendothelium. The invasion of both monocytes and lymphocytes into the arterial intima under ES conditions was not completely prevented by applying the antibody in doses of 2 mg/kg body wt. Mononuclear cells were observed either migrating through or already lying beneath an intact endothelial lining. However, the number of the subendothelially located mononuclear cells was significantly lower than that in the control animals and was approximately one third of the value established in the saline-injected animals (Figure 4; one-sided Wilcoxon test, \( p < 0.05 \)). Even increasing the antibody dose to 3 mg/kg body wt was not effective in abolishing emigration of monocytes and lymphocytes. In two rabbits a dose of 3 mg/kg body wt resulted in 7±2 intimal mononuclear cells, which was in the same range as in the animals injected with 2 mg/kg (i.e., 6±4 mononuclear cells/100 ECs). Plasma cells were not seen within the intima of the stimulated arteries of the MAb-treated animals.

**Smooth Muscle Cell Migration Into the Intima**

Responses to two sessions of ES were characterized not only by the invasion of leukocytes into the subendothelium but also by SMC migration from the media into the intima. Thus, in the carotid arteries of control animals exposed to the described stimulation schedule, after 36 hours SMCs were frequently seen in the process of sending pseudopods through pores of the internal elastic lamina and spreading out into the intima (Figure 5). These SMCs exhibited ultrastructural features of the intermediate phenotype (i.e., clearly visible myofilaments in the peripheral zone, an enlarged perinuclear space free of myofilaments but enriched with mitochondria, and rough endoplasmic reticulum). In some instances, the nucleated part of the SMC had already reached the intima where leukocytes had predominated thus far. The proportion of muscle cells within the entire subendothelial cell population amounted to 39±10%.

In the MAb-treated rabbits SMC movement into the intima was not prevented despite reduced accumulation of leukocytes (Figure 4). It is important to note that migrating SMCs were often found in areas where monocytes/macrophages had accumulated in the intima.

**Total and Differential Leukocyte Counts in Peripheral Blood**

Total leukocyte counts of ES rabbits treated with 2 mg MAb 60.3/kg body wt were compared with those of animals injected with saline (Figure 6). In the control animals a moderate increase in the number of peripheral white blood cells was established 12 hours after implantation of the electrodes and the first saline injection, indicating an inflammatory reaction to the surgical procedure of electrode placement. Leukocyte counts, however, returned to baseline levels at 36 hours (3.0±1.6×10^3/ul). In animals given MAb 60.3 there was marked leukocytosis at all times studied (maximally, 19.8±2.3×10^3/ul at 24 hours), showing a significantly different course from animals not treated with MAb (quadratic analysis of covariance, \( p < 0.05 \)).

With regard to the different leukocyte subsets (Figure 7), a cell type-specific effect of both MAb treatment and time was determined by using multway analysis of variance (\( p < 0.01 \)). In particular, the relative amounts of PMNs (i.e., juvenile and mature forms together) increased within the first third of the examination interval in both experimental groups. However, in the MAb-treated animals the rise in the percentages of the juvenile PMNs was more pronounced (Figure 7). They reached a level of 20±5% at 12 hours, which is significantly different from the respective value in the control group (6±3%, \( p < 0.05 \)). This difference in the amount of juvenile PMNs between MAb-treated and control animals was maintained over the entire period studied. Both total and differential leukocyte counts revealed that MAb administration resulted in marked neutrophilia, which was at least partly based on an increased mobilization of juvenile PMNs.

**Discussion**

Using the model of electrically induced intimal thickening in the rabbit carotid artery, we have demonstrated that 1) MAb 60.3 completely abolished the initial emigration of PMNs as well as their accumulation within the intima, 2) MAb 60.3 only partially inhibited the invasion of mononuclear cells, and 3) the reduced infiltration of leukocytes did not prevent SMC migration into the intima.

The abolishment of PMN accumulation in the ES intima by MAb 60.3 suggests that PMN adherence and/or extravasation are primarily CD18-mediated processes. This finding confirms in vitro data, which showed that MAb 60.3 inhibited adhesion of stimulated human PMNs to human EC monolayers, and it is in accordance with a number of results from in vivo studies in which the antibody profoundly hindered neutrophil emigration in response to inflammatory stimuli (reviewed in Reference 36). Our data were obtained by a
FIGURE 5. Photomicrographs of smooth muscle cells (SMC) migrating from the media into the intima of electrically stimulated carotid arteries from control rabbits. Panel A: Medial smooth muscle cell belonging to the intermediate phenotype is sending pseudopods (arrowheads) toward and into the intima. Panel B: Smooth muscle cell protrusions (arrows) are extending into the intima next to a macrophage (MØ). Panel C: Nucleated portion of a smooth muscle cell has reached the intima. E, endothelium; IEL, internal elastic lamina. Bars=1 μm.
we should mention that solely placing Teflon cuffs current, as done in previous studies, resulted in
of either CD11a/CD18, CD11b/CD18, or CD11c/CD18
might result in enhanced adhesiveness. In this context
tributed by the application of electrical fields, which
initial leukocyte adhesion to the endothelium was ap-
smaller luminal extension than with ES. Moreover,
circumferential intimal thickening with significantly
be speculated, as shown for a variety of membrane
sticking of PMNs is mediated by neutrophil-dependent
endothelial surface. We do not yet know whether ICAM-1 and/or ICAM-2, which have been suggested to function as endothelial counterreceptors of CD11a/CD18 and CD11b/CD18 in other systems, account for PMN extravasation into the arterial intima after ES. It is also unknown whether the enhanced sticking of PMNs is mediated by neutrophil-dependent and/or endothelium-dependent mechanisms. But it may be speculated, as shown for a variety of membrane receptors, e.g., concanavalin A and Fc receptors, that the neutrophil adhesion glycoproteins could be redistributed by the application of electrical fields, which might result in enhanced attractiveness. In this context we should mention that solely placing Teflon cuffs around carotid arteries without application of electrical current, as done in previous studies, resulted in circumferential intimal thickening with significantly smaller luminal extension than with ES. Moreover, initial leukocyte adhesion to the endothelium was approximately 10 times lower. This seems to indicate that electrical current influences either the expression of adhesive molecules on the cell’s surface or the affinity of CD11/CD18 heterodimers for their counterreceptors.
With respect to eosinophils and basophils, MAb 60.3 was not effective in preventing their invasion into the stimulated intima. This may be explained either by the inability of the antibody in the doses used here to saturate all CD18 binding sites on these granulocytic cells or by alternative adhesion pathways that do not involve molecules of the CD11/CD18 complex. Human eosinophils and basophils but not neutrophils were recently shown to constitutively express the integrin receptor VLA-4α and to adhere to human umbilical vein ECs by recognizing the cytokine-induced endothelial ligand VCAM-1. Thus, rabbit homologues of VLA-4 and VCAM-1 may contribute to the recruitment of eosinophils and basophils into the electrically induced intimal thickening. The documented partial inhibition of the extravasation of mononuclear cells also reflects a significant CD18-independent mechanism of both monocyte and lymphocyte emigration that is not abolished by MAb 60.3. This result is consistent with in vitro findings of CD18-independent monocyte binding to endothelial monolayers under different conditions. Likewise, in the rabbit model of inflamed peritoneum, the CD18 MAb was only partially effective in inhibiting mononuclear leukocyte accumulation (R. Winn et al, unpublished observations). Together with these data, we suspect that the residual mononuclear leukocyte migration in our model is mediated by receptor–ligand systems different from those involving CD11/CD18. Interaction between VLA-4, which is present on eosinophils and basophils as well as on resting lymphocytes and monocytes, and its counterreceptor VCAM-1 on activated vascular endothelium was demonstrated to be at least partly responsible for mononuclear leukocyte adherence in other systems. Recently, an ATHERO-ELAM, highly homologous to human VCAM-1, was reported to be expressed on the endothelium that covers atherosclerotic lesions of hypercholesterolemic as well as of Watanabe heritable hyperlipidemic rabbits. A leukocyte-adhesive function of these inducible endothelial surface antigens was suggested to contribute to mononuclear leukocyte recruitment during atherogenesis. It is intriguing to raise the hypothesis

![Figure 6](image-url)
The fact that SMCs occurred within the intima of the MAb 60.3–treated rabbits, despite the reduced accumulation of mononuclear cells, leaves open the question of what role mononuclear cells play in SMC migration. From our results we can only conclude that processes that induce SMC movement toward the intima are sufficiently stimulated, although mononuclear cell infiltration is diminished. Monocytes/macrophages, frequently observed in close association with SMCs that penetrate pores of the internal elastic lamina in the early lesions of our model, could provide mediators for SMC migration. A secretory product of monocytes/macrophages, platelet-derived growth factor, for instance, was shown to have chemotactic activity for SMCs.24-61-63 Other mechanisms, however, may also be involved in regulating the migratory behavior of SMCs (e.g., releasing chemotactants from ECs64-65 or generating tissue-type plasminogen activator66). Tools capable of eliminating the CD18-independent portion of mononuclear cell emigration will help elucidate the contribution of these cells to SMC migration.

In conclusion, the model of the ES rabbit carotid artery has proven to be suitable for investigating 1) the mechanisms underlying the enhanced recruitment of leukocytes during intimal thickening formation and 2) the link between leukocytes that invade the arterial intima and vascular SMC migration. Essential prerequisites for this study were the presence of a morphologically intact endothelium and the speed of SMC migration into the intima, which proved even faster than after balloon catheter injury.67 Present work provides evidence that the leukocyte adhesion complex CD18, recognized by MAb 60.3 and in conjunction with the corresponding endothelial counterreceptors, accounts for both neutrophil and mononuclear cell emigration. For mononuclear leukocytes, however, an inhibited extravasation with CD18 MAb was only partly achieved, indicating the fact that complete elimination of PMN emigration failed to prevent SMC migration excludes PMNs as initiators of this key event in the development of intimal lesions. This finding adds new information to the understanding of SMC behavior in experimental intimal thickening and, possibly, in atherogenesis.

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References


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