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Costimulatory molecules in human atherosclerotic plaques: an indication of antigen specific T lymphocyte activation

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Abstract

Atherosclerotic plaques contain inflammation, composed largely of macrophages and lymphocytes. A proportion of lymphocytes shows signs of activation, but the question arises whether they are activated in an antigen specific way. The expression of costimulatory molecules-receptors that provide accessory signals during antigen-specific activation is a prerequisite for such a condition. This aspect of inflammation in atherosclerotic lesions has not been investigated. Human arterial segments with diffuse intimal thickening, fatty streaks and atherosclerotic plaques were studied with immuno-single and double staining methods. Macrophages and T lymphocytes were stained with CD68 and CD3, respectively, and pan-B cell markers CD19 and CD22 were also used. Costimulatory molecules B7-1 and B7-2, together with their common ligand CD28, and CD27 with its ligand CD70, were stained with specific monoclonal antibodies. The results show that most T lymphocytes were CD27 positive and that only a subpopulation of these (5–15%) was positive also for B7-1, CD28 and CD70. Macrophages expressed B7-1, B7-2, CD28 and CD70, while macrophages positive for CD28 and CD70 have not been reported yet. The expression of costimulatory molecules was most pronounced in the superficial layers at the fibrous cap, but decreased towards the lipid core. This study shows, therefore, that atherosclerotic plaques provide costimulatory signals generally accepted as a prerequisite for adequate T cell stimulation. In addition, this study reveals that only approximately 5–15% of the lymphocytes appears actively involved in the inflammatory reaction. © 1997 Elsevier Science Ireland Ltd.

Keywords: T cell stimulation; Immune mechanisms in atherosclerosis; Immunocytochemical methods; Inflammation and atherosclerosis

1. Introduction

In recent years cellular and humoral immune mechanisms have been considered to play a role in atherogenesis [1–3]. Activated (HLA-DR⁺) macrophages and HLA-DR⁺/IL-2R⁺ T lymphocytes are present in fatty streaks and fully developed atherosclerotic plaques [3–5]. Expression of MHC class II antigens by macrophages in atherosclerotic lesions suggests that these cells present antigen to T lymphocytes. The close

apposition between macrophages and T lymphocytes, with specialized membrane contacts, supports this concept [3,6]. Moreover, the frequency of HLA-DR⁺ T lymphocytes in atherosclerotic lesions is increased as compared to peripheral blood lymphocytes, and the same is true for the activation markers VLA-1, CD26, CD38, and CD45RO [4]. Recently, Stemme et al. [7] demonstrated that T lymphocytes cloned from atherosclerotic plaques recognize oxidized low density lipoprotein. In addition, it was demonstrated that T lymphocytes in atherosclerotic plaques proliferate [8]. What remains unknown as yet is whether the activated lymphocytes present in atherosclerotic plaques are acti-

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vated locally or whether they have been recruited as such [9].

Antigen specific T cell activation is a process which depends on the interaction of the T cell receptor (TCR) with antigens (peptides) presented by MHC molecules and the interaction of costimulatory molecules with their ligands. Activation of T lymphocytes without appropriate costimulation leads to T cell anergy or apoptosis [10,11]. Costimulation is not antigen specific nor MHC restricted and is mediated by several distinct cell surface molecules expressed by antigen presenting cells (APC) and by lymphocytes. In this respect, interactions involving B7 molecules and their ligands are considered of major importance [12]. B7-1 (CD80), B7-2 (CD86) and the recently described B7-3 are costimulatory molecules and members of the immunoglobulin superfamily [11]. Counterstructures of these molecules are CD28 and CTLA-4. In vivo, B7-1 and B7-2 are expressed by human blood dendritic cells, and in vitro by activated, but not by resting monocytes [13,14]. Resident macrophages do not express B7-1, but in granulomatous inflammation macrophages are B7-1⁺ [13]. B7-1 and B7-2 are often co-expressed by APC, but their expression may be differentially regulated by different cytokines [15]. With respect to the cellular distribution of B7-3, thus far, little is known. CD28 is expressed by T-cells, B cells and thymocytes [12]. Many in vitro studies have shown the importance of these molecules in the process of T cell activation. Binding of B7-1 to CD28 on T cells, or cross-linking of CD28 with monoclonal antibodies leads to an augmentation of several T cell functions such as proliferation, cytokine production, adhesion or cytotoxicity [11]. In addition, B7-CD28 interactions are important for lymphocyte activation and they play a role in differentiation process of primed T cells to type 1 and type 2 [16].

Another costimulatory molecule extensively studied and known to play a role in T cell activation is CD27, a member of the tumor necrosis factor (TNF) receptor family (see for review [17]). CD27 is a 120-kDa transmembrane protein composed of disulfide-linked 55 kDa monomers and expressed by a subpopulation of T cells, medullary thymocytes, B cells, and NK cells [18–20]. In vitro results have shown that the expression of CD27 on CD45RO⁺ cells decreases within a time span of approximately 2 weeks upon activation [17,21]. CD27–CD27 ligand (CD70) interactions play a critical role during T cell activation, as well as T cell dependent B cell IgG synthesis [22]. CD70 is expressed on activated, but not on resting T and B lymphocytes, as well as on stromal cells in the thymic medulla [23].

Hence, costimulatory molecules are essential for specific activation of T cells, thus resulting in effective induction, modulation, and amplification of T cell-mediated immunity. However, the question remains whether costimulatory molecules are expressed within

atherosclerotic lesions. Therefore, we examined the presence of the costimulatory molecules B7-1, B7-2, CD28, CD27 and CD70 in different stages of atherosclerosis, namely diffuse intimal thickening, fatty streaks and atherosclerotic plaques.

2. Material and methods

2.1. Tissue specimens

The study is based on 33 arterial segments which included the left coronary artery, carotid arteries and the descending aorta. Samples were obtained at autopsy (13 patients). The ages ranged from 46–83 years with a mean of 65.5 ± 12.5 years.

Specimens were collected within 2–9 h after death. Septic and immunosuppressed patients were excluded from the study. The specimens were snap-frozen in liquid nitrogen.

Cryostat sections, stained with hematoxylin and eosin were used to verify the nature of the sampled specimens. On the basis of the histology three types of lesions were recognized: (i) normal arterial wall with diffuse intimal thickening only ($n = 8$), characterized as a flat intimal thickening due to fibrous tissues; (ii) fatty streaks ($n = 9$), characterized as elevated lesions with focal accumulation of foam cells in the intima; and (iii) atheromatous plaques ($n = 16$) characterized by a core of atheromatous debris encapsulated by fibrous tissue.

2.2. Monoclonal antibodies and conjugates

Antibodies used in this study are listed in Table 1, together with their subtypes, specificities, and origin. For immunohistochemical doublestainings monoclonal antibody EBM-11 (CD68) was biotinylated with D-biotinyl-*E*-amidocaproic acid *N*-hydroxysuccinimide ester (Boehringer, Mannheim). Biotinylated goat anti-mouse Ig (GAM-BIO), alkaline phosphatase (AP)-conjugated goat anti-rabbit Ig (GAR-AP), horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (GAM-HRP), AP-conjugated goat anti-mouse Ig (GAM-AP), AP-conjugated streptavidin (Strept-AP), rabbit anti-FITC and Strept-ABComplex/HRP were obtained from Dakopatts (Glostrup, Denmark). HRP-conjugated swine anti-goat Ig (SAG-HRP) and AP-conjugated swine anti-goat immunoglobulins (SAG-AP) (Biosource Int.). β -Galactosidase-conjugated streptavidin (Strept-GAL) was from Boehringer.

2.3. Immunohistochemistry

Serial cryostat sections (5 μ m) were cut, fixed in cold acetone (4°C) and stored at -20°C . Single staining on the sections was performed using the Strept-ABCom-

plex technique [24]. In short, sections were successively incubated with appropriate, predetermined optimal dilutions of the different antibodies, GAM-BIO, and Strept-ABComplex/HRP. Finally, peroxidase activity was visualized using H_2O_2 as substrate and 3-amino-9-ethyl carbazole (AEC) (Sigma Chemical Co., St. Louis, MO) as chromogen.

The basic concept for the immunoenzyme double staining techniques has been described previously [25,26]. Its detailed performance was dependent on the combination of the primary antibodies. (i) Double stainings with CD3 were performed using a combination of one FITC labeled antibody (CD3-FITC) and one unlabeled primary antibody against one of the costimulatory molecules. According to this protocol, sections were first incubated with the unlabeled antibody, followed by GAM-BIO and Strept-ABComplex. After a blocking step with normal mouse serum, sections were incubated with the FITC-labeled antibody, followed by rabbit-anti-FITC, and GAR-AP. (ii) Double stainings with CD68 were performed using a combination of one biotinylated antibody (EBM-11/biotin) and one unlabeled primary antibody against one of the costimulatory molecules. Sections were first incubated with the unlabeled antibody followed by GAM-HRP and SAG-HRP. After a blocking step with normal mouse serum, sections were incubated with the biotinylated antibody followed by strept-AP. Finally, enzyme activities were detected: first AP activity with naphthol-

AS-MX phosphate as substrate and chromogen Fast Blue BB (Sigma) in blue, and HRP with AEC in red, as described above. (iii) Double staining with CD68 and CD27 were performed as follows. Sections were first incubated with unlabeled CD27 followed by GAM-AP and SAG-AP. After a blocking step with normal mouse serum, sections were incubated with the EBM-11/biotin followed by strept-GAL. Finally, enzyme activities were visualized: first GAL activity with ferro-ferricyanide and X-GAL (Boehringer) in turquoise, and AP activity with the New Fuchsin kit (Dakopatts) in red. Sections of vessel walls where the specific antibody had been omitted or replaced by isotype matched irrelevant antibodies, both for single and double staining procedures, served as negative controls. For the latter, two sections were obtained, each representing one half of the double staining technique. As positive controls cryostat sections of human hyperplastic lymphoid tissue (palatine tonsil tissue), freshly obtained after surgery, were used.

2.4. Quantification of immunohistochemical results

In immunodouble stains using CD3 as pan-T cell marker, the entire section was screened at a magnification of $\times 400$, and the number of T cells expressing costimulatory molecules was counted, and expressed as a percentage of the total number of $CD3^+$ cells.

Table 1
Monoclonal antibodies used in this study

Antibody	Clone and subtype	Specificity	Supplier
CD3	SK7, IgG1	pan-T	BD
CD19	HD37, IgG1	pan-B	Dako
CD22	SHCL-1, IgG2b	pan-B	BD
CD27	CLB CD27-1, IgG2a	Ligand of CD70	CLB
CD28	CLB-CD28-1, IgG1	Ligand of B7-1/2	CLB
CD28	CD28.2, IgG1	Ligand of B7-1/2	Immunotech
CD68	EBM-11, IgG1	Macrophages	Dako
CD70	2F2, IgG1	Ligand of CD27	CLB*
CD70	HNE51, IgG1	Ligand of CD27	Immunotech
CD80 (B7-1)	Dal-1, IgG1	Ligand of CD28	CLB*
CD86 (B7-2)	ITT2, IgG1	Ligand of CD28	Pharmingen
—	IgG1	Isotype control	Pharmingen
—	IgG2a	Isotype control	Pharmingen
—	IgG2b	Isotype control	Zymed

BD, Becton Dickinson (Mountain View, California, USA); CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands); Dako, Dakopatts (Glostrup, Denmark); Immunotech, Coulter-Immunotech (Marseille, France); Pharmingen (San Diego, CA, USA); Zymed (San Francisco, CA, USA).

* Personal gift from Dr R.A. van Lier.

3. Results

All lesions studied contained T lymphocytes ($CD3^+$) and macrophages ($CD68^+$) in highly variable proportions, but B cells ($CD19^+$, $CD22^+$) were not found. Expression of costimulatory molecules was highly variable, but once classified according to the type of lesion distinct patterns surfaced. There were no differences in this respect between lesions obtained from coronary arteries, carotid arteries or from the aorta. The media was not studied. The salient features of the combined staining procedures between costimulatory molecules and T lymphocytes are shown in Fig. 1. Those of macrophages are illustrated in Fig. 2. Isotype matched controls were all negative (data not shown).

3.1. Diffuse intimal thickening ($n = 8$)

Scattered mononuclear cells expressing CD27 and CD28 were found in the intima with diffuse intimal thickening. Double staining with CD3 showed that 60–80% of the T lymphocytes in DIT lesions were $CD27^+$. CD28 was occasionally observed ($< 5\%$) on T lymphocytes. No $CD70^+$ and $B7-1^+$ and $B7-2^+$ T lymphocytes were encountered.

Fig. 1. Micrographs of an atherosclerotic plaque in the aorta. (A) Immunodouble staining for T cells (CD3: blue) with CD27 (red) shows doublestaining of the majority of T cells present in purple; (B) CD3 (blue) combined with CD70 (red). The majority of the CD3⁺ T cells are CD70⁻. The insert shows a CD70⁺/CD3⁺ T cell (arrow). (C) CD3 (blue) together with CD28 (red). Most CD3⁺ cells are CD28⁻. The insert shows CD3⁺ T cells in purple (arrows). Single stained red cells are CD28⁺ macrophages as confirmed by CD68 positivity (see Fig. 2C). (D) CD3 (blue) double stained with B7-1 (red). Only an occasional T cell is B7-1⁺. (A, B, C, D $\times 350$; insert B $\times 875$, insert c $\times 350$).

Fig. 2. Micrographs of an atherosclerotic plaque in the aorta. Immunodouble staining for macrophages. (A) CD68 (blue) with B7-1 (red). There is decreased expression of B7-1 on macrophages near the atheroma (asterisk). (B) CD68 (blue) combined with CD70 (red). Note decreased expression of CD70 on macrophages near the atheroma (asterisk). (A, B $\times 175$).

Macrophages, of which only few had a foam cell morphology, showed CD28⁺ membrane staining. Moreover, all macrophages showed reactivity with anti-CD70, anti-B7-1 and B7-2, as defined by immunodouble staining with anti-CD68. CD27 was not expressed by macrophages.

3.2. Fatty streaks ($n = 9$)

Immunodouble stainings with CD3 showed expression of CD27 by a majority of T cells (60–80%), but only approximately 5–10% of all CD3⁺ cells were CD28⁺. The number of CD3⁺/B7-1⁺ cells varied

between 5 and 15%; an occasional T cell expressed CD70. B7-2 was not observed on T lymphocytes.

A proportion of CD68⁺ macrophages was CD28 positive (Fig. 3a). Control staining procedures in which either CD68 or CD28 was replaced with TBS/BSA further endorsed this observation (Fig. 3b, c). In addition, Fig. 4 shows that staining with CD28 of macrophages was observed with both anti-CD28 monoclonal antibodies. Occasionally CD27⁺ macrophages were observed. B7-1, B7-2 and CD70 were expressed by all macrophages including foam cells. The two anti-CD70 antibodies yielded similar results.

3.3. Atherosclerotic plaques (*n* = 16)

The expression pattern of costimulatory molecules on both T cells and macrophages in atherosclerotic plaques was similar to that observed in fatty streaks, but the pattern of distribution within the plaque differed. Positive cells were seen throughout the fibrous cap, but large accumulations were always seen in the shoulder regions of the plaque. At these sites most macrophages and T cells expressing costimulatory molecules were found in close apposition. Moreover, the expression of B7-1, B7-2 and CD70 on macrophages was most abundant in the superficial region of the fibrous cap, but decreased or was completely absent on necessary macrophages bordering the atheromatous core, an observation confirmed by double staining with CD68.

4. Discussion

This is the first study that shows that T lymphocytes and macrophages in human atherosclerotic plaques express costimulatory molecules generally considered a prerequisite for antigen specific T cell activation. This novel observation is of major importance, since for an appropriate lymphocyte-mediated immune response not only Ag/MHC-TCR interactions are needed, but also additional signals provided by costimulatory molecules [3].

Both B7-1, B7-2, and CD70 were expressed on macrophages in all three types of lesions. This indicates that in all stages of atherosclerosis, macrophages provide the signals necessary for an antigen specific T cell response. To our knowledge, the presence of CD70⁺ macrophages has not been documented as yet. The *in vivo* distribution of CD70 so far reported was limited to B cells in germinal centers, stromal cells in the thymic medulla, and scattered T cells in tonsils, skin and gut [23]. The expression of B7-1, B7-2 and CD70 molecules was most pronounced on macrophages in the superficial layers of the intima. It is of interest that atherosclerotic plaques with a distinct lipid core showed a gradual decrease in the expression of these costimula-

tory molecules towards the atheroma. Macrophages immediately adjacent to the atheroma did not express these molecules. It has been shown previously [27] that macrophages in the fibrous cap of the plaque show a phenotypic shift towards the lipid core. It appears that the expression of costimulatory molecules follows this pattern. These observations thus suggest that macrophages within the more superficial layers of the fibrous cap may act as immunocompetent cells, whereas those at the site of the lipid core have lost this capacity and merely serve as scavenger cells.

The finding of CD28⁺ macrophages is also new and, to the best of our knowledge, not observed in any other inflammatory reaction. The expression of CD28 on macrophages was confirmed by positive staining with an other CD28 antibody. The question thus arises whether this is a phenomenon specific for macrophages in atherosclerotic plaques, or whether CD28 staining could be due to shedding of CD28 from lymphocytes with subsequent binding to B7 receptors on macrophages.

In this context one also may consider the fact that macrophages are phagocytes and, hence, that the expression of CD28 or CD70 on these cells is due to phagocytosed CD70 and/or CD28 initially present on (apoptotic or necrotic) T lymphocytes. However, this explanation is unlikely, because phagocytosed cells are degraded within the phago-lysosomal compartment, with breakdown of proteins, and because the number of T cells which express CD28 and CD70 within the plaques is low and certainly could not account for the abundant expression on macrophages.

Another important issue that arises from this study is the observation that the inflammatory process, associated with atherosclerotic lesions, consists of two populations of T lymphocytes. A minority of T cells (approximately 5–15% of the CD3⁺ cells) expresses B7-1, CD28 and CD70, while the majority (> 70%) are CD27⁺. From *in vitro* studies it is known that activated T cells are able to express B7 molecules [28]. B7-1⁺ T cells have been observed also in other inflammatory diseases; for instance, approximately 10–30% of the T cells in synovial membranes in rheumatoid arthritis are B7-1⁺ [29,30]. Even higher percentages of B7-1⁺ T cells are reported in psoriasis and mycosis fungoides [31]. It has been suggested that B7-1⁺ T cells are capable of autocrine costimulation via the CD28 activation pathway [29]. We did not find B7-1 positive T cells in DIT specimens, indicating that the T cells in these specimens were not (sufficiently) activated for B7-1 expression. On the other hand, it should be noted that the number of T lymphocytes present in DIT is very low. Hence, when only a small percentage of these T cells is B7-1 positive, their finding becomes almost fortuitous.

The expression of CD28 by T lymphocytes *in situ* appears to differ from that in the circulation. Approxi-

Fig. 3. Micrographs of an atherosclerotic plaque in the aorta. Shown are (A) the CD68/CD28 (blue/red resp) double staining, and the controls for the double staining with CD68 (B) and CD28 (C). In B and C, the same staining protocols were performed, only CD28 and CD68 (respectively) were replaced with TBS (A, B $\times 350$).

Fig. 4. Same aortic plaque as shown in Fig. 3. Serial sections stained with two different Mab's against CD28. (A) CLB (CLB CD28-1), and (B) Immunotech (CD28.2). Note similar staining patterns on the cell membranes (A, B $\times 350$).

mately 90% of the T cells in the peripheral blood are CD28⁺ whereas only a minority of T cells in atherosclerotic plaques is CD28⁺ [32]. The relatively low numbers of B7-1⁺ and CD28⁺ T lymphocytes in atherosclerotic plaques resembles chronic inflammatory disease such as rheumatoid arthritis rather than acute inflammatory skin diseases such as allergic dermatitis [33].

The high number of CD27⁺ T cells in atherosclerotic plaques contrasts with the low number expressing CD28. It is well known that CD27 is expressed mostly on naive (CD45RA⁺) T cells, but less so on memory (CD45RO⁺) T cells. Prolonged stimulation, moreover, will further reduce the expression of CD27 [34]. The observation that the vast majority of T lymphocytes in atherosclerotic lesions expresses CD27 suggests that only a small number of T lymphocytes is actively involved in the inflammatory process. This is in accordance with the results obtained by Stemme et al. [4]. They found that approximately 10% of the T lymphocytes in atherosclerotic lesions show signs of recent T cell activation, as judged from the expression of IL-2R positivity. One may speculate, therefore, that lymphocytes in atherosclerotic plaques are essentially of two populations. On the one hand, the largest population made up of 70–90% of the T cells which are not activated, and have a CD27⁺, CD70[−], CD28[−], B7-1[−] phenotype. These cells may represent recirculating and/or bystander cells. On the other hand, there is a population of T cells present, approximately 10%, with a CD27[−], CD70⁺, CD28⁺, B7-1⁺ phenotype. These cells may be recruited from the former population, for instance via local antigen specific stimulation. It is this particular phenotype, therefore, that should be capable of secreting cytokines (e.g. IFN- γ) and modifying various processes, such as smooth muscle cell proliferation and metalloproteinase activity, thus affecting the histomorphology of atherosclerotic plaques. As yet, there is no explanation why the number of activated T cells in atherosclerotic lesions is so low. One explanation could be that the relatively low numbers of activated T lymphocytes present in lesions are due to activation related cell death [35].

The present observations do not unequivocally prove that antigen specific stimulation of T cells takes place. However, these results combined with the observation that interleukin 2 receptor positive T cells are present in atherosclerotic lesions, and to a similar extent, make it likely that antigen specific stimulation indeed occurs. This concept is supported also by the observation that some T cell clones isolated from human atherosclerotic plaques recognize oxidized low density lipoproteins which is widely considered as an important risk factor in atherosclerosis. The present observations, therefore, provide a basis for further functional studies.

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