**Basic Research**

Neovascularization in Human Coronary Arteries With Lesions of Different Severity

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**Introduction and objectives.** Endothelial function can be modulated by growth factors produced by activated smooth muscle cells, inflammatory cells and plasma products that infiltrate the lesion. The aim of this study was to quantify neovessels in human coronary arteries with atherosclerotic lesions of different severity and analyze their relationship with inflammatory cell and plasma product infiltrates.

**Patients and method.** We studied 60 coronary arteries from patients who underwent heart transplant. Cellular markers (smooth muscle cell, monocyte/macrophage), the presence thrombin/prothrombin and expression of vascular endothelial growth factor (VEGF) were analyzed and quantified by conventional histology, immunohistochemistry and image analysis techniques.

**Results.** Neovessels were detected in advanced lesions, and a positive correlation was observed with the degree of vessel remodeling, monocyte/macrophage infiltration and lipid deposition. Smooth muscle cells were the main producers of VEGF in both the intima and media layers of advanced lesions. In these lesions thrombin/prothrombin-positive areas colocalized with activated smooth muscle cells.

**Conclusions.** The presence of neovessels in coronary arteries correlated with inflammatory cell infiltration, lipid deposition and thrombin/prothrombin content. VEGF expression was mainly associated with smooth muscle cells, indicating a key role of these cells in the modulation of endothelial cell function.

**Key words:** Angiogenesis. Atherosclerosis. Smooth muscle cell. VEGF.

**See editorial on pages 947-8**

Partly financed with a grant from the Fundación Española del Corazón (1999-2000).

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Received, 25 September 2002. Accepted for publication, 12 June 2003.

**Neovascularización en arterias coronarias humanas con distintos grados de lesión**

**Introducción y objetivos.** Los factores de crecimiento producidos por las células musculares lisas activadas, las células inflamatorias y especies moleculares plasmáticas infiltradas en la lesión pueden modular la función endotelial. El objetivo de este estudio ha sido cuantificar la presencia de neovasos en lesiones ateroscleróticas de arterias coronarias humanas con diferentes grados de lesión en relación con la infiltración de células inflamatorias y moléculas del plasma.

**Pacientes y método.** Se estudiaron 60 arterias coronarias procedentes de pacientes sometidos a operaciones de trasplante cardíaco. Por técnicas de histología convencional, inmunohistoquímica y análisis de imagen se analizaron y cuantificaron los marcadores celulares (célula muscular lisa, monocito/macrófago), la presencia de lípidos, de trombina/protrombina y los valores de expresión del factor de crecimiento endotelial de origen vascular (VEGF).

**Resultados.** Se detectaron neovasos en lesiones avanzadas y se observó una correlación positiva con el grado de intrusión, la infiltración de monocitos/macrófagos y el contenido lipídico. Las células musculares lisas fueron las principales productoras de VEGF, tanto en la íntima como en la media de las lesiones avanzadas. En estas lesiones se observó colocalización de zonas con un alto contenido de trombina/protrombina, con células musculares en estado activado.

**Conclusiones.** La presencia de neovasos en las lesiones de las arterias coronarias se correlaciona con el contenido de células inflamatorias, de material lipídico y de trombina/protrombina. La expresión de VEGF se asocia principalmente a las células musculares lisas, lo que indica un papel clave de estas células como moduladoras de las células endoteliales.

**Palabras clave:** Angiogénesis. Aterosclerosis. Célula muscular lisa. VEGF.

**Introduction**

Atherosclerosis is a systemic, multifactorial disease involving different risk factors. Multiple growth factors, cytokines and other substances produced by endothelial cells, smooth muscle cells (SMC),
monocytes/macrophages and T lymphocytes control the inflammatory response and cellular proliferation produced in atherosclerotic lesions. The result of the interaction of these factors is a fibroproliferative response that makes atherosclerotic lesions develop. One of the first elements affected during the genesis of atherosclerotic lesions is the endothelium; in fact, endothelial dysfunction is a common denominator in the atherogenic process.7-9 The endothelium also plays a key role in the formation of new blood vessels (neovascularization) which takes place in advanced atherosclerotic plaque.10,11

The formation of new vessels is activated by different growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), whose expression may increase in response to different triggers.12-14 Vascular endothelial growth factor is an angiogenic factor—mitogenic for endothelial cells—that increases vascular permeability and modulates thrombogenicity.15 In the vascular wall, and especially in atherosclerotic lesions, growth factors produced by SMC, such as VEGF, can modulate endothelial function and gene expression linked to the activation of migration and the proliferation of endothelial cells during angiogenesis.16 In addition to these factors, generated in situ by cells that interact in atherosclerotic lesions, thrombin retained by the extracellular matrix, where it remains functionally active,17 seems to play an important role in the regulation of the process. This role of thrombin is based on its ability to promote the activation of vascular cells such as SMC and endothelial cells.

Angiogenesis studies have been done in vitro, in experimental models—mainly with transgenic animals—and in human arteries from autopsies.14,15 However, we do not know of any studies that have systematically analyzed the neovascularization process in atherosclerotic lesions in human coronary arteries obtained from hearts immediately after their extraction during transplant operations. The aim of this study was to quantify the presence of neovessels in coronary lesions of different severity and study growth factor expression potentially associated with such neovascularization.

In the present study on human coronary arteries, we observed significant formation of neovessels in advanced lesions only where the expression of VEGF was highly significant and, in quantitative terms, mainly associated with SMC. We observed colocalization of areas with high thrombin/prothrombin content and active SMC (Ki67-positive). These cells may play a role in the formation of mature neovessels.

PATIENTS AND METHOD

Obtaining and preparing tissue samples

Human coronary arteries were obtained from hearts extracted during transplant operations. Segments approximately 2 cm long were taken from the ostium of the right coronary, circumflex and anterior descending arteries. They were processed immediately after the heart was extracted by fixation in formaldehyde, cryopreservation in sucrose and embedding in OCT (Miles Inc.). All specimens were kept at −80 °C until analysis. One group of samples, after being fixed in formaldehyde, was embedded in paraffin with an automatic tissue processor (Shandon-Elliot).

Frozen and paraffin-embedded samples were then cut into consecutive serial sections 5 μm wide with a cryostat (JUNGCM 3000, Leica) or microtome (Supercut, Reichert).

The arteries were subdivided into fragments for VEGF analysis and randomly assigned to three groups which were processed independently by fixing with formaldehyde, freezing in liquid nitrogen or embedding in paraffin. Immunostaining for VEGF did not distinguish between cellular areas and acellular areas in the samples fixed in formaldehyde. In addition, staining of the cell markers was masked because of the nonspecificity of VEGF staining, thus preventing the identification of cell types. Staining for VEGF in nitrogen-frozen samples was positive in the tunica media and intima. However, the low contrast between cell areas and zones rich in extracellular matrix prevented the clear identification of vascular structures in the intima. It was difficult to observe colocalization of VEGF in cells with double immunostaining. Thus, we chose the paraffin-embedding method to analyze VEGF because of its higher specificity.

Histopathologic and immunohistochemical staining

Staining was done with Masson’s trichromic stain18...
to identify vascular structures and with Oil Red-O (ORO)\textsuperscript{19} to determine lipid deposition. Lesions were then classified according to the criteria of the American Heart Association (AHA).\textsuperscript{20,21}

**Alkaline phosphatase method**

This method was applied with conventional techniques\textsuperscript{22} to look for the following markers: HAM56, prothrombin, \(\alpha\)-SM actin, and CD34 (Table 1).

**Avidin-biotin-peroxidase method**

This method was applied with conventional techniques\textsuperscript{23} to detect VEGF and Ki67 (Table 1). In double immunostaining with VEGF and a cellular marker, we used the avidin-biotin-peroxidase method (ABC Kit, Vector Labs.) for VEGF, and the alkaline phosphatase method (Vector Labs.) for complementary labeling.

**Fluorescence method**

We used this method with conventional techniques\textsuperscript{24} to label von Willebrand factor (vWF) and \(\alpha\)-SM actin (Table 1).

**Image analysis**

The preparations were viewed with a transmitted light and fluorescence binocular microscope (Vanox AHBT3, Olympus). For low magnification we used a binocular magnifying glass (\(\times 25\) magnification). The area of the media (Am), intima (Ai) and lumen (Al) was measured with Visilog software. The percentage of stenosis and vessel remodeling (VR) was calculated with the following formulas:

\[
\text{Stenosis (\%)} = \frac{\text{Ai}}{\text{Ai} + \text{Al}} \times 100
\]

\[
\text{VR (\%)} = \frac{\text{Ai}}{\text{Ai} + \text{Am}} \times 100
\]

Area is expressed in mm\(^2\), and the degree of stenosis and intrusion is expressed as a percentage.\textsuperscript{25,26}

**Quantification of lipid and macrophage area**

Lipid deposition (ORO staining) and monocyte/macrophage (stained with HAM56 marker) content were quantified by capturing five fields per section at \(\times 200\) magnification. A computer program was developed with a built-in subroutine for calculating the percentage of the area of intima occupied by the marker studied. This was done by calculating the percentage ratio of stained area (positive area) to the total area, multiplied by 100.

**Presence of neovessels in lesions**

The areas stained with von Willebrand factor were captured at \(\times 200\) magnification from a section selected at random from those incubated with the combination of anti-von Willebrand factor antibody (vWF) and anti-smooth muscle \(\alpha\)-actin (\(\alpha\)-SM actin) antibody. The area occupied by these neovessels was determined, and the ratios of surface area occupied by neovessels in each area of the wall was calculated.

Neovessel density (D) was calculated with the following formula:

\[
D = \frac{\text{ANv} \times (\text{Am} + \text{Ai})}{100}
\]

where ANv is the area occupied by the neovessels.\textsuperscript{27}

**TABLE 1. Primary antibodies used in immunochemistry**

<table>
<thead>
<tr>
<th>Antigen Recognized</th>
<th>Type</th>
<th>Clone</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Analytical technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAM56</td>
<td>Monoclonal</td>
<td>HAM56</td>
<td>1/50</td>
<td>DAKO</td>
<td>FA-POD</td>
</tr>
<tr>
<td>VEGF</td>
<td>Polyclonal</td>
<td>Rabbit IgG</td>
<td>1/400</td>
<td>Santa Cruz Biotech</td>
<td>ABC-POD</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>Polyclonal</td>
<td>Rabbit Ig fraction</td>
<td>1/100</td>
<td>DAKO</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>CD34</td>
<td>Monoclonal</td>
<td>QEEnd/10</td>
<td>1/50</td>
<td>Novocastra</td>
<td>FA-POD</td>
</tr>
<tr>
<td>(\alpha)-SM actin</td>
<td>Monoclonal</td>
<td>1A4</td>
<td>1/50</td>
<td>DAKO</td>
<td>Fluorescence FA-POD</td>
</tr>
<tr>
<td>Ki67</td>
<td>Monoclonal</td>
<td>MIB-1</td>
<td>1/50</td>
<td>Immunotech.</td>
<td>ABC-POD</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Polyclonal</td>
<td>Sheep IgG</td>
<td>1/100</td>
<td>DAKO</td>
<td>FA-POD</td>
</tr>
</tbody>
</table>

Markers: CD34 and vWF (endothelium and neovessel markers); HAM56 (monocyte/macrophage marker); \(\alpha\)-SM actin (smooth muscle cell (SMC) marker; Ki67 (proliferation marker). Methods: ABC, avidin-biotin; AP, alkaline phosphatase; POD, peroxidase.
Statistical analysis

The results were expressed as the mean±SEM (standard error of the mean) and analyzed with Statview™ software (Abacus Concepts). Between-group comparisons were made with the Mann Whitney U-test, and regression analysis was done with the Spearman non-parametric test. Differences were considered significant at $P<.05$.

RESULTS

Characteristics of patients

We analyzed coronary arteries from 20 Caucasian patients (90% males and 10% females) undergoing transplants. Their ages ranged from 18 to 63 years and they were selected over a period of 18 months (from October 1999 to March 2001). Table 2 presents the risk factor distribution in these patients according to the underlying pathology—dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (IC). The presence of hyperlipidemia (88%), arterial hypertension (67%) and diabetes (11%) was prominent in IC compared to the absence of such risk factors in patients with DCM. All the patients with IC had a history of old myocardial infarction.

Classification of lesions

Sixty lesions were studied, corresponding to 60 coronary arteries. Table 3 shows the distribution of lesions according to risk factors. In general, advanced lesions were found in patients with IC (24 of 25 lesions, types IV to VIII), whereas early lesions were found in patients with non-ischemic DCM (32 of 35 lesions, types I to III). Figure 1 shows typical examples of such lesions. Spearman’s test showed that the severity of lesions, which entails an increase in the degree of stenosis, correlated positively with monocyte/macrophage infiltration ($r=0.536; P=.0001$), and was greatest in type VI lesions. Severity later decreased in the final stages (type VII and VIII), and with decreases in lipid deposition ($r=0.647; P=.0001$).

Neovascularization in human atherosclerotic lesions

In early lesions (types I and II) neovessels were not detected. They appeared in intermediate lesions (type III), and increased in number up to the type VI stage, but decreased in advanced lesions (type VII and VIII). Neovessels were found around the lipid core, the media and between the media and intima. Double immunolocalization studies showed that the areas surrounding neovessels are rich in inflammatory cells, especially monocytes/macrophages. In general terms, these are areas where SMC ($\alpha$-SM actin marker) are more numerous. Figure 2 shows a typical example of an advanced lesion with high neovessel content. Table 4 shows the areas occupied by neovessels in different types of lesion. Spearman’s test showed a positive correlation between neovessel content and the degree of stenosis ($r=0.740; P=.0001$), intrusion ($r=0.758; P=.0001$), monocytes/macrophage infiltration ($r=0.537; P=.0001$), lipid deposition ($r=0.663; P=.0001$) and the presence of thrombin/prothrombin ($r=0.650; P=.0001$).

VEGF expression in lesions

The paraffin-embedding method was the most effective for analyzing VEGF. This factor colocalized with cellular areas and identified perivascular areas in
the vascular endothelium and in intimal neovessels (Figure 3B and F). The vasa vasorum of the adventitia also showed positive staining for VEGF. Staining for this factor colocalized mainly with the SMC of the media and intima (Figure 3C, D and G), and also with endothelial cells (Figure 3E) and macrophages (Figure 3H). Areas rich in collagen were negative for VEGF. As seen in Figure 3, mature neovessels are formed by a single layer of endothelial cells surrounded by smooth muscle (positive for α-SM actin).

**Expression of proangiogenic molecules: thrombin**

Thrombin/prothrombin were not detected in arteries with intimal thickening (TAI) (Figure 4C), but increased in the intima as the lesion developed, with highest levels in type VI lesions (Figure 4D). In type II and III lesions these molecules were associated with the presence of monocytes/macrophages, SMC and lipid material. In advanced lesions, they were seen in association with SMC, monocytes/macrophages and extracellular matrix. A high thrombin/prothrombin content was also associated with activated (Ki67-positive) SMC (Figure 5).

**DISCUSSION**

In this study we investigated the presence of neovessels and VEGF in relation to markers of lesion progression in human coronary arteries.

Twenty patients were included, of whom 9 had IC and 11 had DCM. Most advanced lesions were found in patients with IC, and the most prevalent risk factors were hyperlipidemia and hypertension. Only one of the patients of the 20 included in the study had diabetes, as this is a contraindication for heart

**TABLE 4. Content of neovessels in atherosclerotic lesions**

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>TAI</th>
<th>I-II</th>
<th>III</th>
<th>IV-V</th>
<th>VI</th>
<th>VII-VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neovessels</td>
<td>0.00% (n=12)</td>
<td>0.00% (n=16)</td>
<td>0.06% (n=7)</td>
<td>0.27% (n=6)</td>
<td>0.69% (n=7)</td>
<td>0.53% (n=12)</td>
</tr>
</tbody>
</table>

Neovessels expressed as the percentage of coronary artery wall area (Am+Ai) occupied by endothelial markers.

*P<.05 vs TAI and I-II. **P<.05 vs TAI, I-II, III, and IV-V. *P<.05 vs TAI, I-II, and III. TAI indicates thickening of arterial intima.
and the proteins under study.

In the lesions analyzed here, the presence of intimal neovessels correlated with the percentage of stenosis, intrusion, inflammatory cell content, lipid material content and the presence of thrombin/prothrombin. The presence of lipids gradually increased in lesions up to type VI, these being the richest in neovessels. Previous studies have associated the presence of neovessels with thrombotic complications and, in fact, these are the type VI plaques most often associated with thrombotic complications. The colocalization of neovessels with monocytes/macrophages also suggests an important role for these cells, which produce angiogenic factors that can stimulate the formation of neovessels.

In vitro and in vivo studies have shown that VEGF is the most important angiogenic factor. However, its analysis has involved the use of a variety of processing and fixation methods, usually in human arteries taken from autopsies in which the extraction time ranged from 3 h to 16 h post mortem. The results of these studies may differ from ours to some extent, because with immunohistochemical methods, the way samples are obtained and processed can significantly affect the results. We found that the paraffin-embedding method was the most specific for analyzing VEGF in human coronary arteries removed immediately after extraction of the heart during transplant operations. Vascular endothelial growth factor was expressed in lesions mainly by SMC, in agreement with previous studies in various human arteries. In intimal neovessels, VEGF colocalized with SMC and endothelial cells. Expression was associated with vascular cells, but also with monocytes/macrophages. Some researchers have detected VEGF in the extracellular matrix, in areas close to SMC, and in macrophages; a more generalized pattern of distribution has also been reported. We detected significant VEGF expression only in the extracellular matrix in samples fixed in formaldehyde, a procedure in which staining for was not very specific.

In addition to autocrine factors such as VEGF, generated by vascular cells in situ, other plasma products infiltrating the vascular wall, such as thrombin, can promote the angiogenic process. Thrombin modulates metalloproteinase activity and has mitogenic potential in endothelial cells and SMC. It can also release «hijacker» growth factors for matrix components, and thus can also indirectly promote the process of angiogenesis. In advanced lesions, we observed colocalization of areas with high thrombin/prothrombin content and active (Ki67-positive) SMC.

Although many studies have found transplantation. Early lesions were mainly found in patients with DCM who did not present these risk factors. The age of patients with IC was significantly higher than those with DCM. In fact, no patient less than 45 years old presented advanced lesions or evidence of thrombotic complications in their coronary arteries. The study was done in arteries processed immediately after the heart was surgically removed. This approach might provide information closer to the in vivo situation than the analysis of autopsy specimens, since the latter are normally obtained several hours post mortem. In addition, protein immunoreactivity can become altered in samples from autopsies, and the cellular contents can be released, making it difficult to locate cell markers only in the extracellular matrix in samples fixed in formaldehyde, a procedure in which staining for was not very specific.
neovascularization in the intima of atherosclerotic lesions in samples from patients who died from acute complications, its pathophysiological role is controversial. Our study showed that in atherosclerotic lesions in patients who have received heart transplants, the level of neovascularization is low, even in advanced lesions in patients with previous episodes of ischemic cardiomyopathy. This could be due to the strict pharmacological regimen practiced with these patients. Thus, excessive angiogenesis would favor plaque rupture and the development of acute events. This hypothesis is currently being evaluated in our laboratory.

**Study limitations**

Given that this study was based on stable patients on the waiting list for heart transplantation, we note that it does not provide data on patients with acute phase disease or diabetes. Obviously, both groups are of interest and will be the focus of future research.

**CONCLUSIONS**

We detected neovessels from the type III stage onward, and these neovessels were most numerous in type VI lesions. Neovessels were found in the intima as well as in adjoining areas between the intima and media, in areas rich in lipid material, and in inflammatory cells. Neovessels were found almost exclusively in patients with IC who had advanced lesions associated with a higher incidence of risk factors, such as hypertension and hyperlipidemia. In these lesions VEGF expression was significant, and
was associated in quantitative terms mainly with SMC.

Acknowledgment

The authors would like to acknowledge the ongoing collaboration of the heart transplant team of the Cardiology and Cardiac Surgery Division of the Hospital de la Santa Creu i Sant Pau. This work was made possible by funds initially provided by Marató-TV3 for Heart Disease in 1995, which enabled the founding of the tissue bank, and by the Fondo de Investigaciones Sanitarias (FIS99/0907) and the Plan Nacional (SAF2000-0174). The post-doctoral work of Josep O. Juan-Babot was supported by a grant from the Fundación Española del Corazón 1999-2000. The authors would like to acknowledge the continuing support of the Fundación de Investigación Cardiovascular-Catalana Occidente.

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