Angiopoietin 2 Induces Cell Cycle Arrest in Endothelial Cells: A Possible Mechanism Involved in Advanced Plaque Neovascularization

Cristina Calvi, Patrizia Dentelli, Marco Pagano, Arturo Rosso, Marco Pegoraro, Sara Giunti, Giovanni Garbarino, Giovanni Camussi, Luigi Pegoraro, Maria Felice Brizzi

Objective—To characterize the molecules and the mechanisms regulating the neoangiogenetic process in advanced atherosclerotic plaques.

Methods and Results—Western blot and immunofluorescence analysis of atherosclerotic specimens demonstrated that unlike neovessels from early lesions that expressed vascular endothelial growth factor (VEGF) and angiopoietin1 (Angio1), vessels from advanced lesions expressed VEGF and angiopoietin 2 (Angio2). Moreover, only few neovessels from advanced lesions showed a positive immunostaining for proliferating cell nuclear antigen. Angio1-elicited and Angio2-elicited intracellular events in endothelial cells (EC) demonstrated that while Angio1 triggered Erk1/Erk2 mitogen activated protein kinases (MAPK) and Akt activation, Angio2 (50 ng/mL) induced STAT5 activation and p21waf expression and increased the fraction of cells in G1. Both Angio2-mediated events were abrogated by expressing a dominant negative STAT5 construct. Consistent with the expression of Angio2 in neovessels of advanced lesions a transcriptionally active STAT5 was detected. Moreover, co-immunoprecipitation experiments revealed the presence of a STAT5/Tie2 molecular complex in neointima vessels from advanced, but not from early, lesions.

Conclusions—In advanced lesions, the activation of the Tie2-mediated STAT5 signaling pathway may negatively regulate vessel growth.

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Key Words: angiogenesis • atherosclerosis • growth factors • STATs • signal transduction

Atherosclerosis is viewed as a progressive inflammatory disease in which lipids, inflammatory cells, and smooth muscle cells accumulate into the arterial wall and contribute to plaque growth.1,2 In addition, recent results demonstrate that neovascularization, induced by in vivo administration of vascular endothelial growth factor (VEGF), also contributes to the development of atherosclerotic lesions.3 Consistently, in vivo developing primary lesions might be considered as underperfused and dependent on neovessel formation for continued growth.4 Angiogenesis in mature tissues is composed of a series of cellular responses including activation of EC proliferation by angiogenic factors, such as VEGF, fibroblast growth factor (FGF), and angiopoietins (Angio) and formation of tube-like structures and vessel stabilization.5 Compared with the developmental angiogenesis, the role of angiopoietins in pathological angiogenesis is less understood. The angiopoietin family consists of four members, Angio1 to 4.6–8 Angio1 and 4 stimulate their receptor tyrosine kinase Tie2, whereas Angio2 and 3 by binding to Tie2 antagonize Angio1 signaling.6–8

Signal transduction pathways via Tie2 and VEGF receptors (VEGFR) have been extensively examined.9,10 In particular, Korpelainem et al showed that Tie2 and VEGFR1 overexpression triggers the phosphorylation of the signal transducer and activator of transcription (STAT) 3 and STAT5.11 STAT5 belongs to the family of transcriptional factors, which, on activation, binds to the receptor via their src homology 2 domain (SH2).12 Recruited STATs, dimerize, translocate to the nucleus, and activate transcription of target genes mainly involved in regulation of cell cycle progression.12,13

Cell cycle phases are coordinated by regulatory proteins denoted as cyclins and cyclin-dependent kinases (CDKs).14 Phase-specific cyclin/CDK complexes confer specificity and orderly progression through the cell cycle. Initially, cyclin D/CDK4 or cyclin E/CDK2 complexes, in cooperation with proliferating cell nuclear antigen (PCNA), coordinate DNA duplication by regulating the transition through the G1 and S phase.15 Furthermore, cell cycle progression is regulated by the expression of cyclin-dependent kinase inhibitors (CKIs) such as p27kip1 and p21waf, which bind to CDKs and prevent their activation14,15

Accumulating evidences sustain the notion that transcriptional factors by regulating CDKs and CKIs expression
modulate cell cycle progression.\textsuperscript{16,17} STAT5 has been reported to regulate the CKI \textit{p21}\textsuperscript{18} expression in different settings\textsuperscript{18,19} that include Tie2-mediated and VEGFR1-mediated signals.\textsuperscript{11} This observation is consistent with the finding that Tie2\textsuperscript{26–28} and VEGFR\textsuperscript{20} activation did not trigger proliferating signals in ECs.

In a condition such as atherosclerosis, intimal angiogenesis occurs as a part of the adaptive changes known as vascular remodeling.\textsuperscript{21} It has been clearly established in rodent models that inhibition of intraplaque neoangiogenesis significantly reduced plaque growth.\textsuperscript{22} However, clinical studies and in vitro experiments demonstrated that risk factor for coronary artery disease are associated with a significant impairment in adaptive vascular growth\textsuperscript{23,24} and that neovascularization is more abundant in restenotic atheromas compared with similar-size primary lesions,\textsuperscript{25} suggesting that events strictly associated with the late stage of plaque evolution may modify the angiogenic response inside the neointima.

In the present study, we concentrated on the analysis of the mediators and of the signaling events associated with the neoangiogenic process in the advanced stage of the atherosclerotic process. We found that in contrast with the active angiogenic pattern characteristic of the early lesions, in the advanced ones the neoangiogenesis may be hampered as suggested by the low expression of PCNA, the high expression of Angio2, and the activation of the Tie2-mediated STAT5 signaling pathway.

Methods

Reagents

Nitrocellulose filters, horseradish peroxidase-conjugated protein A, molecular weight markers, (α-\textit{32}P)dCTP, Poly(dIdC):poly(dIdC), the chemiluminescence reagent (ECL) (Amersham; Braunschweig, Germany), anti-STAT5, anti-p21\textit{red}, anti-Angio2, anti-Angio1, anti-VEGF (Santa Cruz Biotechnology; Heidelberg, Germany), FITC-conjugated and RTIC-conjugated anti-rabbit, nonimmune mouse or rabbit IgG, anti-phospho-STAT5, anti-phospho-Erk\textendash{}1/\textendash{}Erk\textendash{}2 MAPK, anti-phospho-Akt, anti-Akt antibodies (New England Biolabs; Beverly, Mass), FITC-conjugated anti-CD105, anti-CD105 antibodies (Tecnogenetics S.R.L.; Milan, Italy), and anti-PCNA (Dako; Glostrup, Denmark) were used. Angio1, Angio2, and VEGF were from R&D Systems; 4’,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) was from Sigma.

Human Arterial Samples

The institutional review board of the hospital approved the study. Human nonatherosclerotic and atherosclerotic arteries (coronary and carotid arteries) were obtained from informed patients who underwent transplantation and carotid endarterectomy or from autopsy and were fixed and embedded in paraffin. By histology, we classified these into nondiseased (n=6), diffuse intimal thickening (type I, n=10), fatty streaks (type II, n=10), atheromatous plaques (type Va, n=15), fibromuscular plaques (type Vc, n=27), eroded plaques (type VI, n=15), and ruptured plaques (type VI, n=8) according to the American Heart Association histological criteria.

Cell Cultures and Transfections

Human endothelial cells (EC) were isolated and characterized as described.\textsuperscript{19} ECV304 cell line, a bladder cancer-derived cell line,\textsuperscript{26} has been reported to express many endothelial surface antigens,\textsuperscript{27,28} among them is Tie2 (Brizzi et al unpublished data). These cells were transfected with the dominant negative STAT5 (ΔSTAT5) construct\textsuperscript{13} by the lipofectin methods. Expression of the ΔSTAT5 protein was analyzed by Western blotting and the positive clones were tested by electrophoretic mobility shift assay.\textsuperscript{29}

Flow Cytometry

EC or ECV304 cells were stimulated in serum-free medium with Angio1 or Angio2 (50 ng/mL) for 18 hours and then fixed with 70% ethanol. After digestion with RNase, DNA was stained with propidium iodide and analyzed with a flow cytometer.

Immunoprecipitation and Western Blot Analysis

Neointima from fresh samples of advanced atherosclerotic specimens or intima from nonatherosclerotic specimens were homogenized in cold DIM buffer (50-mmol 7l pipes, pH 6.8, 100 mmol/L NaCl, 5 mmol/L MgCl\textsubscript{2}, 300 mmol/L sucrose, 5 mmol/L EGTA, 2 mmol/L sodium ortovanadate plus 1% Triton X-100 and a mixture of protease inhibitors/1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 0.15 U/mL aprotinin, and 1 mg/mL pepstatin A) immediately after endarterectomy or surgical procedures in the course of organ transplantation. EC were unstimulated, Angio1-stimulated, or Angio2-stimulated (50 ng/mL or 800 ng/mL) for different time intervals and then lysed. Equal amounts of proteins (500 μg) were immunoprecipitated with the indicated antibodies, and immunocomplexes were bound to protein-A-Sepharose beads. Bound proteins were eluted and processed as previously described.\textsuperscript{19,30}

Preparation of Nuclear Extract and Gel Retardation Assay

Nuclear extracts from normal and from neointima of advanced tissues were prepared as described by Sadowski and Gilman;\textsuperscript{31} 10 μg of nuclear proteins/sample were used. The double-stranded p21\textsuperscript{SE2} oligonucleotide sequences, which are the potential STAT5 binding sites in the p21\textsuperscript{SE2} promoter region,\textsuperscript{18,19} were used. Labeling of oligonucleotides and gel retardation reactions were performed as previously described.\textsuperscript{19}

Immunofluorescence

Atherosclerotic and nonatherosclerotic specimens from carotid formalin (10%) fixed and embedded in paraffin were stained with the indicated antibodies or DAPI and processed as described.\textsuperscript{19,30} Nuclear localization of PCNA was analyzed by confocal microscopy. Confocal microscopy was performed on a Leica TCS SP2 model confocal microscope (Heidelberg, Germany) using a ×63 magnification lens and 488-nm argon laser.

Northern Blot Analysis

Cytosplasmic RNA was isolated from EC by guanidinium thiocyanate/acid phenol–chloroform extraction.\textsuperscript{32} Northern blot analysis was performed as previously described.\textsuperscript{29} Filters were hybridized to P random-labeled DNA probes corresponding to p21\textsuperscript{SE2} and β-actin and were processed as previously described.\textsuperscript{29}

Results

PCNA and VEGF Expression in Vessel From Early and Advanced Lesions

The rate of intraplaque neovessel formation during plaque evolution was first evaluated by analyzing the expression of PCNA, the well known DNA duplication marker.\textsuperscript{15} To this end, an immunofluorescence with an anti-CD105 antibody recognizing the endothelial membrane antigen endoglin; and with an anti-PCNA antibody, immunofluorescence was performed in early and advanced lesions. A positive signal for PCNA was detectable in vessels from early lesions (Figure 1, available online at http://atvb.ahajournals.org). Conversely, the majority of the vessels inside the neointima of advanced lesions were negative for PCNA immunostaining. The nu-
On the contrary, Angio2, already detectable in normal vasculature and early lesions, was abundantly expressed in advanced lesions. Whereas Angio1 is produced by mesenchymal cells surrounding neovessels,^8 Angio2 has been shown to be produced in autocrine fashion by EC. We found that, also in neointima, a positive immunostaining for Angio2 was detectable in CD105 positive cells (Figure 1B and 1C). As negative control, nonimmune rabbit IgG was used in place of primary antibodies (Figure 1D). It is well established that Angio2 and Angio1 exert their biological effects by binding to Tie2. Western blot analyses of lysates from normal vessels and from early and advanced lesions were performed. As shown in Figure 1A, unlike in normal vasculature and in vessels from early lesions, high levels of Tie2 were detected in advanced lesions.

Angio1 and Angio2 Initiate Distinct Signaling Pathways in EC
The high expression of Tie2 in advanced lesions that expressed both Angio1 and Angio2 prompted us to analyze, by in vitro experiments, biochemical and biological events associated with Tie2 engagement by both ligands. It has been shown that overexpression of Tie2 induces p21waf expression in a STAT5-dependent mechanism. To identify as Angio1, Angio2, or both, the ligands responsible for STAT5 activation, Angio1 and Angio2, were evaluated for their ability to activate Tie2 and STAT5. A dose–response curve demonstrated that Tie2 activation was clearly detectable at the concentration of 50 ng/mL of Angio2 (data not shown). As shown in Figure 2A, both Angio1 and Angio2 at the concentration of 50 ng/mL induced Tie2 activation in EC. Moreover, a 90-kDa phosphoprotein corresponding to the immunoprecipitated phospho-STAT5 (positive control) also could be detected in the anti-Tie2 immunoprecipitates only in Angio2 treatment. Consistently, Angio2, but not Angio1, triggered STAT5 activation (panel B of Figure 2). Panel C of Figure 2 shows the kinetic of STAT5 activation in response to Angio2. Besides the activation of STAT5, activation of MAPK and Akt was evaluated in EC treated with Angio1 or Angio2. Unlike Angio2, Angio1 triggered both Erk1/Erk2 MAPK and Akt activation (Figure IIIA and IIIB, available online at http://atvb.ahajournals.org). The apparent discrepancy between our results and data obtained by Kim et al^26 on Angio2-mediated Akt activation can be caused by the doses of Angio2 used, (50 ng/mL versus 800 ng/mL). When EC were stimulated with high doses (800 ng/mL) of Angio2, Akt but not STAT5 became phosphorylated, indicating that physiological and high concentrations of Angio2 trigger discrete signaling pathways possibly leading to different biological responses (Figure IIIC, available online at http://atvb.ahajournals.org). Conversely, high doses of Angio1 did not trigger STAT5 activation (data not shown).

Angio1-Induced and Angio2-Induced Cell Cycle Progression
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Angio1, Angio2, and Tie2 Expression in Advanced Lesions
In adult neoangiogenesis, vessel fate is mainly regulated by the interplay between the angiopoietins and VEGF. The observation that, unlike neointima vessels from early lesions, vessels from advanced lesions showed a low expression of PCNA and no differences in the level of VEGF expression led us to evaluate whether the transition from early to advanced lesions could be associated with changes in Angio1/Angio2 expression. The results reported in Figure 1A demonstrated that Angio1 was stably expressed during lesion progression. On the contrary, Angio2, already detectable in normal vasculature and early lesions, was abundantly expressed in advanced lesions. We hypothesize that an unbalanced growth factor release could account for the impaired EC proliferation. Therefore, the high expression of Tie2 in advanced lesions that expressed both Angio1 and Angio2 prompted us to analyze, by in vitro experiments, biochemical and biological events associated with Tie2 engagement by both ligands. It has been shown that overexpression of Tie2 induces p21waf expression in a STAT5-dependent mechanism. To identify as Angio1, Angio2, or both, the ligands responsible for STAT5 activation, Angio1 and Angio2, were evaluated for their ability to activate Tie2 and STAT5. A dose–response curve demonstrated that Tie2 activation was clearly detectable at the concentration of 50 ng/mL of Angio2 (data not shown). As shown in Figure 2A, both Angio1 and Angio2 at the concentration of 50 ng/mL induced Tie2 activation in EC. Moreover, a 90-kDa phosphoprotein corresponding to the immunoprecipitated phospho-STAT5 (positive control) also could be detected in the anti-Tie2 immunoprecipitates only in Angio2 treatment. Consistently, Angio2, but not Angio1, triggered STAT5 activation (panel B of Figure 2). Panel C of Figure 2 shows the kinetic of STAT5 activation in response to Angio2. Besides the activation of STAT5, activation of MAPK and Akt was evaluated in EC treated with Angio1 or Angio2. Unlike Angio2, Angio1 triggered both Erk1/Erk2 MAPK and Akt activation (Figure IIIA and IIIB, available online at http://atvb.ahajournals.org). The apparent discrepancy between our results and data obtained by Kim et al^26 on Angio2-mediated Akt activation can be caused by the doses of Angio2 used, (50 ng/mL versus 800 ng/mL). When EC were stimulated with high doses (800 ng/mL) of Angio2, Akt but not STAT5 became phosphorylated, indicating that physiological and high concentrations of Angio2 trigger discrete signaling pathways possibly leading to different biological responses (Figure IIIC, available online at http://atvb.ahajournals.org). Conversely, high doses of Angio1 did not trigger STAT5 activation (data not shown).

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content. The results shown in Table 1 indicate that the cell population in S and G2/M phases decreased after the cells were subjected to Angio2. On the contrary, treatment of EC with Angio1 led to moderate increase of the cell population in S (30% compared with 26% in the control) and G2/M phases. Moreover, unlike that of Angio1, the effect exerted by Angio2 was accompanied by an increase of 71% (compared with 60% in the control) in the percentage of cells in G0/G1 (Table 1). To dissect the mechanisms involved in these effects, we analyzed the expression of the CKIs p21\textsuperscript{waf} and p27\textsuperscript{kip1}. As shown in Figure 3A, Angio2, but not Angio1, was able to induce p21\textsuperscript{waf} protein expression. Conversely, the expression of p27\textsuperscript{kip1} was not affected (Figure 3A). Consistently, Northern blot analysis demonstrated that Angio2, but not Angio1, induced the expression of p21\textsuperscript{waf} mRNA (Figure 3B).
TABLE 2. Effects of ∆STAT5 Expression on Angio2-Mediated Cell Cycle Events

<table>
<thead>
<tr>
<th>Cell Cycle Phases</th>
<th>Percentage of Cells</th>
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<tbody>
<tr>
<td></td>
<td>Neo</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>G0/G1</td>
<td>52 ± 4.5</td>
</tr>
<tr>
<td>S</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>G2/M</td>
<td>10 ± 2.5</td>
</tr>
</tbody>
</table>

Neovector and ∆STAT5 ECV304-derived clones were unstimulated (control) or stimulated for 18 hours with Angio2 (50 ng/mL), harvested, fixed in ethanol, and evaluated by flow cytometric analysis for the DNA content. Percentage of cells in each phase is shown as the mean of three individual experiments, as determined by ModFit LT software (Verity Software House).

These data suggest that Angio2, by increasing the expression of p21waf, can inhibit cell cycle progression.

**∆STAT5 Expression Abrogates the Effects of Angio2 on p21waf Expression and on Cell Cycle Progression**

To assess the role of STAT5 in regulating p21waf expression, ECV304 cells expressing the ∆STAT5 construct were stimulated with Angio1 or Angio2 and assayed for p21waf expression. As shown in Figure 3C, unlike in neo-vector-expressing cells, in ∆STAT5-expressing cells Angio2 treatment failed to stimulate p21waf expression. Angio1 did not affect p21waf expression in neo-vector-expressing and in ∆STAT5-expressing cells. Moreover, in culture of neo-vector-expressing cells, Angio2 treatment led to a reduction of the percentage of cells in S phase and to an increase of cells in G1 phase (Table 2). Conversely, Angio2 treatment of cells expressing the ∆STAT5 construct led to an increase of the percentage of cells in S phase and to a corresponding reduction of the percentage of cells in G1. These data indicate that Angio2, through STAT5, regulates cell cycle progression.

**STAT5 Is Activated and p21waf Is Expressed in EC of Advanced Lesions**

To evaluate whether the expression of the activated STAT5 and p21waf could be related to the expression of Angio2 in advanced lesions, immunofluorescence analysis was performed on specimens at different stages of lesion progression. A double immunostaining on nonatherosclerotic specimens and on specimens from intimal thickening (n = 10), fatty streak (n = 10), atheromatous plaques (n = 15), fibromuscular plaques (n = 27), and eroded plaques (n = 15) was performed with an anti-CD105 with an anti-phospho-STAT5 or with an anti-p21waf antibody (Figure IVA). Consistent with the absence of vascularity within the intima, no CD105 immunoreactivity could be detected in nonatherosclerotic lesion (data not shown) or in arteries with diffuse intima thickening. The presence of newly formed vessels in the intima of fatty-streak lesions was demonstrated by the CD105 immunoreactivity. Early lesional vessels did not express phospho-STAT5 and p21waf. Conversely, co-localization of phospho-STAT5 and CD105 as well as of p21waf and CD105 were detected in fibromuscular and eroded plaques. The results demonstrated that activation of the transcriptional factor STAT5 and the expression of p21waf in neovessels are strictly associated with the advanced stage of the disease.

**Biochemical Analysis of STAT5 Activation and p21waf Expression in Advanced Lesions**

We have previously shown that the activated STAT5 was almost exclusively expressed by EC. To further confirm the presence of the activated STAT5 and p21waf in neovessels of advanced atherosclerotic lesions, Western blot was performed. The expression of p21waf and of the phosphorylated-STAT5 was detected in advanced lesions. Conversely, the presence of STAT5 immunoreactivity, but not that of phospho-STAT5, in nonatherosclerotic specimens and in specimens from early lesions indicates that the protein is present but not activated in normal vasculature or in the early stage of plaque development. Similarly, no p21waf expression was detected in normal vessels and in early lesions (Figure IV). STAT5 activation was also assessed by electrophoretic mobility shift assay using the p21SIE sequence. Nuclear extracts from advanced lesions, but not from normal vessel, were able to form p21SIE/protein complex. The presence of STAT5 in the complex was demonstrated by the ability of the anti-STAT5 antibody to super-shift the p21SIE binding complex (Figure IVD).

**Activated Tie2 Physically Interacts With STAT5 in Advanced Lesions**

The observation that Angio2, but not Angio1, was able to induce STAT5 activation and p21waf expression in EC led us to evaluate whether the activated Tie2 could interact with STAT5 in vivo. The results of co-immunoprecipitation experiments on lysates from advanced lesions, but not from nonatherosclerotic vessels, depicted in Figure 4A, demonstrated the presence of two bands of phosphoproteins corresponding to 140 and 90 kDa in the anti-Tie2 immunoprecipitates. As positive control, an anti-Tie2 immunoprecipitate from Angio2-stimulated EC was used. The anti-Tie2 immunoblot (lower panel) demonstrated that Tie2 itself was the 140-kDa protein revealed by phosphotyrosine antibody. Moreover, the inability of the anti-phosphotyrosine immunoblot to recognize the 90-kDa band when immunoprecipitation performed in the absence of cell lysate (negative control) confirmed the specificity of the co-immunoprecipitation experiments. Equal amount of proteins from anti-Tie2 immunoprecipitates were separately loaded and immunoblotted with the anti-STAT5 antibody. The results on the left panel (Figure 4) demonstrated that the 90-kDa phosphoprotein co-precipitated by the anti-Tie2 antibody was indeed STAT5. To further confirm this interaction, lysates from early and advanced lesions were immunoprecipitated with an anti-STAT5 antibody and immunoblotted with the phosphotyrosine antibody. The results of panel B (Figure 4) demonstrated that together with the phosphorylated STAT5, the anti-STAT5 antibody co-precipitated a 140-kDa phosphoprotein only in lysates from advanced, but not from early,
Angiogenesis in adult tissues is regulated by the combined effects of negative and positive signals. In this context, VEGF is required to initiate vessel formation, whereas Angio1 is subsequently required for further remodeling and maturation of this initially immature vasculature. After vessel maturation, Angio1 seems to continue to be important in maintaining the quiescence and the stability of the mature vasculature. Disruption of the signals coincides with reinitiation of vascular remodeling in adults. Such destabilization seems to involve the autocrine induction of a natural antagonist of Angio1, termed Angio2. In our study, no changes in VEGF and Angio1 expression were detected during lesion progression, whereas Angio2 expression increased in advanced lesions. It has been shown that VEGF administration can initiate vessel formation in adult animals, leading, in the absence of other factors, to the formation of leaky, unstable vessels. By contrast, Angio1 in vivo administration stabilizes and protects adult vasculature from the damage and leak induced by VEGF. VEGF and angiopoietins apparently recapitulate their developmental role during vascular remodeling in adults. Thus, the presence of both VEGF and Angio1, in the relative absence of Angio2 in early lesions, may parallel adult vessel formation. They guarantee stable and non-leaky vessels and promote plaque growth. By contrast, the presence of Angio2 in neovessels of advanced lesions may initiate vascular remodeling by disrupting cell–cell and cell–matrix contacts, even in the presence of VEGF. As proposed by Lobov et al., after Angio2-mediated destabilization of VE–cadherin junctions, EC become more dependent than usual on VEGF-mediated cell proliferation and undergo growth arrest. The work of Carmeliet et al. shows that the signal transduction by VEGF strictly depends on the integrity of VE–cadherin containing adherent junctions. Consistent with an Angio2-mediated growth arrest, we found that the majority of neointima vessels of advanced lesions showed a negative immunostaining for PCNA. Thus, these observations suggest that in the late stage of plaque evolution, vessels are prevalently quiescent.

The role of Angio2 is still controversial. In early reports, Angio2 has been shown to antagonize Angio1-mediated Tie2 activation; however, when Tie2 was ectopically transfected, Angio2 induced Tie2 activation. Moreover, the observations that high concentrations of Angio2 enhances EC survival and stimulates migration and tube-like structure formation raise the possibility that rather than acting as an antagonist of Angio1, Angio2 can sustain an active role in Tie2-mediated signaling. Signal transduction pathways via Tie2 have been extensively examined; however, in many studies Tie2 was activated in a ligand-independent manner or by high concentrations of Angio2. In the present study, we demonstrate that Tie2 activation in EC can also be elicited by physiological concentrations of Angio1 and Angio2. The characterization of Angio1 and Angio2 as agonists or antagonists was based on the ability of Angio2 to bind Tie2 and to inhibit the pro-angiogenic effect of Angio1; however, several studies on Angio2 functions have suggested a more complex situation. This study shows that Tie2 engagement by Angio1 or Angio2 in EC activates discrete signaling pathways and that physiological or high concentrations of Angio2

lesions. Thus, these data indicate that Tie2/STAT5 interaction only occurs in the late stages of the disease.

Discussion

The role of ischemia in regulating intraplaque neovessel formation has been postulated, suggesting that neoangiogenesis may represent a prerequisite for plaque growth. The role of ischemia in regulating intraplaque neovessel formation has been postulated, suggesting that neoangiogenesis may represent a prerequisite for plaque growth. The role of ischemia in regulating intraplaque neovessel formation has been postulated, suggesting that neoangiogenesis may represent a prerequisite for plaque growth.
can elicit STAT5 activation to control cell growth or Akt phosphorylation to control cell survival, respectively.36

We previously reported that the activated STAT5 and p21waf were almost exclusively expressed in neovessels of advanced lesions.19 Herein we demonstrate that, in contrast with NFκB, which is also expressed in early lesions,47 a positive immunostaining for the activated STAT5 could be detected only in EC of advanced lesions. STAT5 is a pleiotropic regulator of gene transcription activated in response to different stimuli, including cytokines and growth factors.48 Although we could not identify the factors responsible for STAT5 activation in the thickening intima, the co-expression of Angio2, Tie2, and activated STAT5 in advanced lesions suggest a potential role of Angio2 in STAT5 activation. Among the genes transcriptionally regulated by STAT5, p21waf is included.11,18,19 We found a positive immunostaining for p21waf in neovessels from advanced but not from early lesions. Indirect evidence of a causal relationship between STAT5 activation and p21waf expression in the thickening intima is provided by the demonstration that lysates from advanced lesions expressed the activated STAT5 and p21waf, and that nuclear extracts from advanced lesions, but not from normal vessels, were able to bind a STAT5-recognizing sequence on the p21waf promoter to form a complex containing STAT5. This observation and the notion that adventitia and media vessels, which constitute the potential reservoir of postnatal angiogenesis and seem to contribute to the growth of neointima thickening,38 did not express phospho-STAT5 and p21waf (data not shown) suggest that consistent with the low immunoreactivity for PCNA, their expression in neointima vessels of advanced lesions may be associated with perturbation of EC proliferation.

Tie2 belongs to the tyrosine kinase receptor family.10 Tight binding of STATs to activated receptors seems to play a critical role in STAT5 signaling.12,13 We found that in neointima of advanced, but not in nonatherosclerotic, vessels and in neointima of early lesions, Tie2 was activated. Moreover, consistent with the results obtained with Angio2-treated EC, activated Tie2 acquired the ability to physically interact with STAT5. These data sustain the possibility that events strictly associated with the advanced stage of the atherosclerotic process may contribute to Tie2-mediated STAT5 activation and to the increased p21waf expression. We have no direct evidence that Angio1, rather than Angio1, is the Tie2 ligand that, by activating the enzymatic activity of the receptor, coupled to and activated STAT5 and possibly induced p21waf expression. However, the following observations sustain this possibility: (1) Angio1 expression was found in early lesions when neither STAT5 activation nor p21waf expression was detected; (2) Angio2 was prevalently expressed in advanced lesions; (3) Angio2, but not Angio1, induced STAT5 activation and p21waf expression in vitro; (4) Angio2-mediated p21waf expression was abrogated in cells ectopically transfected with the ΔSTAT5 construct; and, more importantly, (5) the Tie2/STAT5 complex could be detected only in advanced lesions.

It has been clearly established in several animal models that intraplaque neoangiogenesis is critical for lesion development and progression;4,22 however, the role of neoangiogenesis in advanced plaques is less understood. In the present study, we show that in contrast with the active angiogenetic pattern observed in the early stage, in the late stage neoangiogenesis may be hampered as suggested by the low expression of PCNA and by the expression of Angio2. In addition, our findings provide evidences for the role of the STAT5 signaling pathway in modulating intraplaque neoangiogenesis.

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References


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