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K. Toutouzas, E. Messaris, M. M. Konstadoulakis, E. Chatzigianni, M. Karayannis, P. S. Davaris, G. Androulakis

Increased gene expression has been found in both animal and human atherosclerotic lesions, mediating cell turnover and lesion development. We investigated the role of p53, bcl-2, c-myc and H-ras genes in atherosclerotic lesions. We examined immunohistochemically the carotid arteries of 86 patients who underwent athrectomy for primary atherosclerosis and 20 normal carotid arteries. C-myc over-expression was seen in 13 (15.1 %) cases and H-ras in 21 (24.4 %) cases of the 86 specimens, while no expression of either gene was found in the normal specimens. P53 and bcl-2 genes were not expressed in either the atherosclerotic or the normal specimens. Six out of 7 patients that co-expressed the c-myc and H-ras genes in their lesions were hypertensive (> 160/95) (p = 0.04). No significant correlation was found between the gene expression and the risk factors or the presence of clinical symptoms. Although, c-myc and H-ras are not independently correlated to one of the risk factors of atherogenesis, their co-expression is significantly increased in hypertensive patients. P53 and bcl-2 were not expressed in either the study or the control group, however, their absence of expression may be important in atherogenesis. J Clin Basic Cardiol 2002; 5: 253–6.

Key words: atherosclerosis, apoptosis, c-myc, H-ras, bcl-2, p53, hypertension

Atherosclerosis is characterized by intimal thickening caused by accumulation of cells, lipids and connective tissue [1]. As in other tissues, cells of cardiovascular tissues can undergo apoptosis. Increased apoptosis has been found in both animal and human atherosclerotic lesions, mediating cell turnover and lesion development. Atherosclerosis is characterized by intimal thickening caused by accumulation of cells, lipids and connective tissue [2]. Smooth muscle cells (SMCs), are the predominant cell type in the atheromatous plaque, and their increased proliferation and migration are pivotal events in the development of occlusive vascular disease [3]. Atherosclerosis is an inflammatory disease [2] so in addition to vascular cells macrophages and T cells are present in atherosclerotic lesions, producing biologically active substances such as the proinflammatory cytokines, tumor necrosis factor, interleukin-1 and interferon-gamma. The proliferation of SMCs is controlled in part by the actions of specific peptide growth factors, as well as by direct cell to cell interaction within the vascular wall [4]. Simultaneous exposure to these cytokines may trigger apoptosis of the vascular smooth muscle cells. The products of apoptosis regulating genes including fas/fas ligand, members of interleukin-1 beta cysteinyl protease (caspase) family, the tumor suppressor gene p53, and the protooncogene c-myc are frequently detected in all human organs and tissues [1]. As in other tissues, cells of cardiovascular tissues can undergo apoptosis. Increased apoptosis has been found in both animal and human atherosclerotic lesions, mediating cell turnover and lesion development. Atherosclerosis is characterized by intimal thickening caused by accumulation of cells, lipids and connective tissue [2]. Smooth muscle cells (SMCs), are the predominant cell type in the atheromatous plaque, and their increased proliferation and migration are pivotal events in the development of occlusive vascular disease [3]. Atherosclerosis is an inflammatory disease [2] so in addition to vascular cells macrophages and T cells are present in atherosclerotic lesions, producing biologically active substances such as the proinflammatory cytokines, tumor necrosis factor, interleukin-1 and interferon-gamma. The proliferation of SMCs is controlled in part by the actions of specific peptide growth factors, as well as by direct cell to cell interaction within the vascular wall [4]. Simultaneous exposure to these cytokines may trigger apoptosis of the vascular smooth muscle cells. The products of apoptosis regulating genes including fas/fas ligand, members of interleukin-1 beta cysteinyl protease (caspase) family, the tumor suppressor gene p53, and the protooncogene c-myc are frequently detected in vascular cells and may play an important role in the pathogenesis and/or progression of atherosclerosis [5].

The present study investigates the role of the p53, bcl-2, c-myc and H-ras expression in patients with atherosclerosis, in order to evaluate the role that these genes have in the pathogenesis and/or progression of this disease and their association with risk factors such as hypertension, smoking, hyperlipidaemia and diabetes mellitus.

Materials and Methods

Arterial Specimens

Atherosclerotic plaques from human carotid arteries with atherosclerosis were obtained from 88 patients who underwent carotid endarterectomy. All the patients’ charts were reviewed to identify information such as age, gender, and presence of risk factors and presenting symptoms. Specimens were obtained from 88 patients, however, 2 patients were excluded from the study, because of incomplete data. Our study group consisted of 60 (69.8 %) males and 26 (30.2 %) females, with a mean age of 65.1 years (median: 65 years, range: 39 to 80 years). Twenty-three (26.7 %) of the patients presented with symptoms, such as transient ischaemic attack, amaurosis fugax and cerebrovascular accident, while 63 patients (73.3 %) were asymptomatic (Table 1).

The degree of carotid stenosis was estimated directly with duplex examination. Peak systolic velocity that was more than 250 cm/sec, end diastolic peak velocity that was more than 100 cm/sec, and internal carotid/common carotid peak velocity ratio more than 1.8 were used to determine a > 75 % stenosis. Additional angiography was performed in several patients to confirm the result of duplex examination. All patients included in the study had carotid stenosis > 75 %.

Twenty samples of normal carotid arteries, obtained from the Department of Forensic Medicine at the University of Athens, were used as control group. All samples were retrieved from persons with an average age of 18 years (range 17 to 19 years) who died in motor vehicle accidents with no past medical history and no atherosclerotic changes in autopsy. The retrieval of study and control specimens was approved by the Ethics Committee of the Hippocration Hospital. All specimens were placed in fresh 4 % paraformaldehyde solution immediately after retrieval. After 2 hours the tissue was transferred to a 30 % sucrose phosphate buffer solution.

<table>
<thead>
<tr>
<th>Risk factor Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus</td>
<td>19 (22.1 %)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>40 (46.5 %)</td>
</tr>
<tr>
<td>Smoking</td>
<td>47 (54.7 %)</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>8 (9.3 %)</td>
</tr>
<tr>
<td>Patients with at least one risk factor</td>
<td>66 (76.7 %)</td>
</tr>
</tbody>
</table>
and then embedded in paraffin. For each tissue specimen, several 4 μm sections were cut. One section from each specimen was stained with hematoxylin and eosin for conventional light microscopy analysis.

Immunohistochemical Analysis
Paraffin tissue sections (5 μm) were mounted on poly-lysine coated glass slides. Paraffin was removed from the slides when heated at 60 °C for 10 minutes and then washed three times in xylene for 10 minutes each. After gradual hydration through graded alcohols, the sections were rinsed in distilled water for 5 minutes. Slides were then incubated for 30 minutes in 0.3 % hydrogen peroxide in methanol to quench endogenous peroxidase activity. The sections were additionally incubated in citrate buffer solution (0.01 mol/L: pH 6.0) for two five-minute cycles in a microwave oven for p53 and bcl-2 antigen retrieval. For localization of the p53, bcl-2, c-myc and H-ras proteins, the sections were incubated for 60 minutes at room temperature with a 1:50, 1:40, 1:10 and 1:15 dilution, respectively, of the monoclonal antibody (Dakopatts, Glostrup, Denmark) and visualized by the indirect avidin-biotin procedure with 3,3-diaminobenzidine tetrahydrochloric as the substrate chromogen. The immunostained slides were then counterstained with hematoxylin for 1 minute.

Microscopically, three sections of the same specimen on each slide were examined by light microscopy. The sections were obtained from macroscopically identified lesions, provided the evaluation of the whole lesion.

At the beginning the ×200 magnification was used, in order to clarify the positive status or not of the specimen and then a median of 5 high power fields (×400) on each section, in order to precisely identify the exact cellular composition that was positive. The apoptotic cells were characterized by the presence of nuclear fragments and pyknotic nuclei. When positive cells were < 10 % or none, the section was considered as negative.

The cell population consisted of smooth muscle cells, macrophages (foam cells, full of lipid material), few monocytes and lymphocytes. The presence of cholesterol clefts, or thrombus in some sections was also taken into consideration. As positive controls for p53, c-myc and H-ras, we used sections from breast carcinomas previously stained positively, while for bcl-2 normal lymph node tissue was used. Negative controls were obtained by substituting the primary antibody with the immunoglobulin fraction of non-immune mouse serum. Negative control sections did not stain. Two independent pathologists evaluated the slides separately, without any knowledge of the clinical data.

Statistical Analysis
The association between the gene expression and the risk factors for developing atherosclerosis, as well as the presence of clinical symptoms was examined. Statistical evaluation of the results was performed using the chi-square test. Differences were assumed to be significant at p < 0.05.

Results
Thirteen of the 86 cases (15.1 %) expressed the c-myc protein (Fig. 1a) and 21 of the 86 cases (24.4 %) expressed the H-ras protein (Figs. 1b, 2). All normal specimens were found negative for c-myc and H-ras staining. None of the examined tissues, atherosclerotic and controls, were p53 or bcl-2 positive.

These findings were examined for any association with known atherosclerosis risk factors, including hypertension, smoking, hyperlipidaemia and diabetes mellitus. C-myc and H-ras overexpression were found to be correlated with the presence of hypertension (p = 0.04). Six out of 7 (85.7 %) patients that expressed both c-myc and H-ras protein had hypertension, while only 34 out of 79 (43 %) patients that did not express any protein or expressed only one had hypertension (Fig. 3). No significant correlation between the single expression of c-myc or H-ras genes and the presence of risk factors for developing atherosclerosis was found (Tab. 2).

Furthermore, no correlation was found between the expression of p53, bcl-2, c-myc and H-ras proteins and the presence of clinical symptoms, such as transient ischaemic attacks and amaurosis fugax (p = 0.184).

Figure 1. a) Positive immunostaining for c-myc gene expression in a mixed cell population consisting of foam cells and smooth muscle cells (×200); b) Positive immunostaining for H-ras gene expression. Giant cells, foam cells and macrophages are evident (×200).

Figure 2. Expression of c-myc and H-ras genes in patients with carotid atherosclerotic lesions.
Discussion

Atherosclerosis is an inflammatory disease characterized by the proliferation of the smooth muscle cells in the arterial wall. The initial cell migration and proliferation that is the result of a vessel injury or of an endothelial dysfunction is believed to be the major cause of this disease [2]. However, the accumulation of cells in the intimate is the sum of cell migration and cell growth. Cell death and alteration of any of these events affects this disease process.

Apoptosis is frequently seen in normal endothelial cells. Still, documented evidence of apoptosis in cardiovascular tissues is limited. In vitro studies first established the principle that apoptosis could in fact be identified in both cardiomyocytes and vascular SMCs [6–8]. Bennet et al. [9] report apoptosis in vascular SMCs in culture; apoptosis was not only seen in cell populations with high proliferative activity (transfected cells constitutively expressing the proto-oncogene c-myc), but also in normal vascular SMCs deprived of serum. Thus, apoptosis is likely to modulate the number of normal and neointimal vascular SMCs in the arterial wall. Possibly as a part of normal vascular wound healing, whereas dysregulated apoptotic mechanisms and inefficient removal of apoptotic bodies may contribute to the progression of the atherosclerotic plaque and increase the severity of the disease. Excessive apoptosis without appropriate phagocytosis may result in the additional macrophage recruitment and the secretion of inflammatory cytokines. This may induce increased cellular migration, proliferation and other disease complicating factors, such as the release of oxidized lipids that exacerbate the severity of atherosclerotic lesions.

There is increasing evidence that the protein encoded by the tumor suppressor gene p53 induces apoptosis [10]. This is a cell cycle regulator involved in DNA repair, DNA synthesis, cell differentiation and apoptosis and may account for the cellularity of atherosclerotic plaques. Furthermore, it was recently found that p53 accumulates in human atherosclerotic tissue. Vascular SMCs from atherosclerotic plaques have an increased sensitivity to p53 mediated apoptosis than SMCs from normal vessels [11]. On the contrary other investigators [12] report the absence of p53 involvement in the pathogenesis of human atherosclerotic lesions. Investigators also report immunopositivity for p53 in athrectomy specimens retrieved from restenotic, but not primary lesions [13, 14]. Their findings are further supported by our study, where none of the specimens examined, atherosclerotic or normal, was p53 immunoreactive. Latest reports [15] demonstrate that the lack of expression of p53 gene increases cell proliferation in atherosclerotic lesions.

The bcl-2 proto-oncogene was originally identified in a human chromosomal translocation that predisposes affected individuals to malignant transformation of immune cells. Increased expression of bcl-2 enhances the cellular survival of immune cells [16], haematopoietic cells [17] and neurons [18]. The bcl-2 product is a membrane-associated protein that suppresses apoptosis in several different cell types. Bennett et al. [19] showed that human vascular SMCs apoptosis is suppressed by the expression of the bcl-2 gene. Whether bcl-2 or bcl-2 family members regulate apoptosis in vascular SMCs in vivo is still debatable. Vascular SMCs express low levels of bcl-2 in vitro and in vivo [11], but this is not universally found [14, 19]. The mechanism of bcl-2 action is unknown, but bcl-2 expression can inhibit apoptosis because of free radicals and lipid peroxidation, both of which are implicated in atherogenesis [20]. Bcl-2 expression was not seen in any of the atherosclerotic arteries or normal carotid arteries examined in our study.

The molecular and biochemical events that drive the SMCs from quiescence to proliferation, a central mechanism to the development of occlusive vascular disease, involve growth factor stimulation and activation of early response genes. One of this cell cycle regulating genes is the proto-oncogene c-myc [21]. Its product is a highly conserved nuclear phosphoprotein, whose expression is closely linked to cellular proliferation and differentiation. Previous studies in rats demonstrated that the expression of c-myc gene may induce apoptosis in vascular SMCs [9]. In our study, the c-myc protein was expressed in 15.1% of the specimens examined.

Ras genes are expressed in the majority of human tissues and have a signal transduction activity. Ras proteins interact with a wide spectrum of regulators and downstream effectors producing different cellular responses, including proliferation, differentiation or apoptosis. Proliferation of SMCs of the arterial wall in response to local injury is an important aetiologic factor of vascular proliferative disorders such as atherosclerosis and restenosis after angioplasty. Ras proteins are key transducers of mitogenic signals from membrane to nucleus in many cell types, including SMCs and macrophages. A key role of ras in vascular SMC proliferation in a rat model was reported by Indolfi et al. [22]. They reported that ras has a major role in SMC proliferation and the local delivery of transforming dominant negative mutants of ras in vivo might prevent some of the acute vascular damage caused by balloon injury. In our study the ras protein was expressed in 21 (24.4%) cases.

Table 2. Correlation of gene expression and risk factors for developing atherosclerosis

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Diabetes</th>
<th>Smoker</th>
<th>Hyper-</th>
<th>Hyper-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mellitus</td>
<td>smoking</td>
<td>tension</td>
<td>lipidaemia</td>
</tr>
<tr>
<td>Myc positive</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Myc negative</td>
<td>17</td>
<td>56</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>P-value</td>
<td>0.72</td>
<td>0.36</td>
<td>0.97</td>
<td>0.95</td>
</tr>
<tr>
<td>Ras positive</td>
<td>3</td>
<td>18</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Ras negative</td>
<td>16</td>
<td>49</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>P-value</td>
<td>0.38</td>
<td>0.81</td>
<td>0.133</td>
<td>0.39</td>
</tr>
</tbody>
</table>
It has been shown that physiological remodelling can occur by apoptosis and cell proliferation in development of ductus arteriosus [23] and in remodelling of vessel mass after birth [24]. The remodelling of the vessel wall also observed in atherogenesis and in the vessels of patients with hypertension is again mediated by the coordinated action of cell proliferation and apoptotic cell death. Furthermore, hypertension is known to induce apoptosis in the vessel media [25] and regression of medial hypertrophy after reduction of hypertension via vascular SMCs’ apoptosis [26]. In our study the expression of the c-myc protein may represent the induction of cell apoptosis [9] in atherosclerotic plaques and the expression of the ras protein may induce cell proliferation [21]. The ‘apoptotic-proliferative’ remodelling was not observed in all the patients with atherosclerosis, or in the control group. Still, a statistically significant (p = 0.04) increase of the c-myc expression role of several genes that are associated in the pathogenesis of atherosclerosis, in order to further understand atherogenesis and to develop new, more effective types of treatment, such as vascular gene therapy [27].

References

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