Transfection of Antisense p53 Tumor Suppressor Gene Oligodeoxynucleotides Into Rat Carotid Artery Results in Abnormal Growth of Vascular Smooth Muscle Cells

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Background—Although loss of activity of an antioncogene, the p53 tumor suppressor gene product, has been postulated to negatively regulate the cell cycle in various cell types.2–4 However, little is known about the role of p53 in the regulation of vascular smooth muscle cell (VSMC) growth. In this study, to clarify the role of p53 in the pathogenesis of restenosis, we examined transfection of antisense p53 oligodeoxynucleotides (ODN) into VSMC in vitro and rat carotid artery in vivo.

Methods and Results—The specificity of antisense p53 ODN was confirmed by a significant decrease in p53 protein. Transfection of antisense p53 ODN into VSMC resulted in a significant increase in DNA synthesis and cell number as compared with sense and scrambled ODN (P<0.01). Importantly, transfection of antisense p53 ODN into intact carotid artery resulted in a significant increase in the ratio of neointima to medial area at 2 and 4 weeks after transfection, accompanied by a significant decrease in p53 protein (P<0.01). Moreover, cotransfection of wild-type p53 plasmid completely abolished neointimal formation induced by antisense p53 ODN. The sustained effect of a single antisense ODN administration was confirmed by the kinetics of ODN in the vessel wall with the use of FITC-labeled ODN.

Conclusions—Overall, the present study demonstrated that loss of p53 by antisense p53 ODN resulted in an abnormal VSMC growth in vitro and in vivo. These results demonstrated the potential contribution of p53 to the pathogenesis of restenosis. (Circulation. 2000;101:1447-1452.)

Key Words: restenosis ■ muscle, smooth ■ apoptosis ■ cells ■ genes

Intimal hyperplasia is the pathological process that underlies restenosis after angioplasty, atherosclerosis, and vascular graft occlusion1 and develops in large part as a result of vascular smooth muscle cells (VSMC) proliferation and migration that are activated by vascular injury.1 The process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle–regulatory genes, which results in mitosis. Especially p53 (p53 tumor suppressor gene) has been postulated to negatively regulate the cell cycle in various cell types.2–4 However, little is known about the role of p53 in the regulation of VSMC. The presence of a functional p53 protein has been implicated as a critical determinant to regulate DNA replication, DNA repair, and programmed cell death.5–8 First, p53 has been shown to induce cell cycle arrest at the G1/S boundary border, related to its ability to induce expression of a cellular gene, WAF1/CIP1/SDI1, which encodes a 21-kDa inhibitor of G1 cyclin–dependent kinases.9 Second, p53 can induce apoptosis through bcl-2–dependent and bcl-2–independent pathways.10,11 Therefore the abnormal progression of the cell cycle seen in cancer is thought to result from the mutation of these negative cell cycle regulatory genes, especially p53.2–3

The importance of cell cycle regulation in VSMC is apparently great, since we and others have previously reported the successful prevention of restenosis after angioplasty using antisense oligodeoxynucleotides (ODN) against cell cycle–regulatory genes, decoy cis element of E2F binding site, and gene transfer of nonphosphorylated Rb (retinoblastoma gene).12–14 Of importance, recent studies have suggested that loss of p53 activity by cytomegalovirus infection might be responsible for the pathogenesis of human restenosis.15–17 From this viewpoint, it is necessary to understand the negative regulation of the cell cycle by p53 in VSMC. This study examined the role of p53 in negative regulation of the cell cycle in the pathogenesis of restenosis, with the use of antisense strategy in vivo. The present study successfully demonstrated in vivo evidence of importance of p53 in the regulation of VSMC growth.
In Vitro Transfection of Antisense p53 ODN Into VSMC

Initially, we tested whether transfection of antisense p53 ODN into VSMC has an effect on VSMC growth. Transfection of antisense p53 ODN resulted in a significant increase in DNA synthesis assessed by $^3$H-labeled thymidine incorporation as compared with untreated and sense p53 ODN-treated VSMC (Figure 1a, $P<0.01$). Of importance, transfection of antisense p53 ODN also resulted in a significant increase in the number of VSMC as compared with sense ODN-transfected VSMC ($P<0.01$, Figure 1b). The specificity of antisense p53 ODN was confirmed by the observation that a marked decrease in p53 protein was only observed with transfection of antisense p53 ODN but not sense p53 ODN or HVJ complex alone (Figure 2).

In Vivo Transfection of Antisense p53 ODN Into Rat Intact Carotid Artery

As shown in Figure 3, successful transfer of antisense p53 ODN by means of HVJ-liposome complex was confirmed by Western blotting of p53 protein. Five days after transfection, Western blots from untransfected intact vessels and intact vessels transfected with sense ODN demonstrated a single band corresponding to p53 protein. Administration of antisense p53 ODN decreased p53 protein but did not affect the level of tubulin protein (Figure 3). Transfection of antisense p53 ODN into intact rat carotid artery resulted in significant decrease in p53 expression as compared with transfaction of HVJ alone or sense ODN ($P<0.01$).

As shown in Figure 4a, PCNA-stained nuclei were observed in the medial layer of blood vessels transfected with antisense p53 ODN at 4 days after transfection, whereas few PCNA-stained nuclei were found in vessels transfected with sense p53 ODN. In addition, untransfected intact carotid arteries also exhibited few PCNA-stained nuclei. Consistent with in vitro experiments, the ratio of PCNA-positive nuclei to total cells in the blood vessels transfected with antisense p53 ODN was significantly increased as compared with those transfected with sense p53 ODN ($P<0.01$, Figure 4b). Next, we examined the effects of antisense p53 ODN on neointimal formation after transfection. As shown in Figure 5, a and b, the vessels transfected with HVJ-liposome complex without ODN, sense p53 ODN, or scrambled p53 ODN exhibited little neointimal formation at 2 weeks after transfection. In contrast, a single administration of antisense p53 ODN resulted in a significant increase in the ratio of neointimal area to medial area (Figure 6). There was no significant difference in medial area among HVJ-transfected, sense p53 ODN transfected, and antisense p53 ODN transfected vessels.
ODN-transfected, scramble p53

ODN-transfected, and anti-sense p53 ODN-transfected vessels (Table 1). Further evidence for the specificity of antisense p53 ODN was provided by the observation that treatment with neither antisense ACE ODN nor antisense thrombomodulin ODN affected neointimal formation (antisense ACE 124 ± 95 μm² vs antisense thrombomodulin 156 ± 101 μm², P > 0.05). In addition, neointimal formation was due to the lack of p53 activity, as cotransfection of wild-type p53 plasmid and antisense p53 ODN completely abolished neointimal formation induced by antisense p53 ODN (Figure 7). Surprisingly, neointimal formation was still observed 4 weeks after a single transfection in blood vessels transfected with antisense p53 ODN (P < 0.01, Figure 8). There was no significant difference in medial area between sense p53 ODN-transfected and antisense p53 ODN-transfected vessels (Table 2).

To confirm the sustained effect of a single intraluminal administration of antisense p53 ODN, we performed in vivo transfer of FITC-labeled ODN into intact carotid arteries by using the HVJ-liposome method. Transfer of FITC-labeled phosphorothioate ODN by the HVJ method resulted in widespread distribution of fluorescence in medial vascular cells even 2 weeks after transfection (Figure 9). The fluorescence was localized primarily in cell nuclei and persisted up to 2 weeks after transfection. Untreated or HVJ complex without ODN-treated vessels revealed no specific fluorescence in the elastic lamina. Intraluminal incubation of free FITC did not result in specific fluorescence in the vessel wall, demonstrating that this fluorescence was specific for FITC-labeled ODN (also see Figure 9).

**Discussion**

A fundamental pathological feature of vascular disease is the abnormal accumulation of cells within the intimal space,

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Medial Area, μm²</th>
<th>Neointimal Area, μm²</th>
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<tbody>
<tr>
<td>HVJ</td>
<td>16 227.797</td>
<td>131 ± 131</td>
</tr>
<tr>
<td>P53-SC</td>
<td>15 487.2062</td>
<td>195.97</td>
</tr>
<tr>
<td>P53-S</td>
<td>16 227.2230</td>
<td>149 ± 110</td>
</tr>
<tr>
<td>P53-AS</td>
<td>16 288.1853</td>
<td>3739 ± 772*</td>
</tr>
</tbody>
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*P < 0.01 vs HVJ, P53-SC, P53-S.
resulting in neointimal formation produced by alterations in the homeostatic balance between cell growth and cell death.\textsuperscript{18} Of importance, loss of p53 activity induced by cytomegaloviral infection might be related to the pathogenesis of human restenosis.\textsuperscript{15–17} However, there is no direct in vivo evidence that loss of p53 might induce restenosis. Thus it is important to understand the role of p53 in the regulation of VSMC. Our in vitro experiments demonstrated that transfection of antisense p53 ODN promoted DNA synthesis and growth of VSMC. Since regulation of the intimal cell population requires a delicate balance between cell influx, cell growth, and cell death,\textsuperscript{19} cells lacking p53 undergo cell cycle progression, resulting in abnormal cell growth. More importantly, in vivo transfection of antisense p53 ODN into intact carotid artery also resulted in a significant increase in neointimal area at 2 and 4 weeks after transfection. Several observations provide evidence that VSMC growth is induced by lacking p53: (1) PCNA-positive cells were only detected in blood vessels transfected with antisense p53 but not sense p53 ODN; (2) neointimal formation induced by antisense p53 ODN was observed both at 2 and 4 weeks after transfection; (3) neointimal formation was only observed in blood vessels transfected with antisense p53 ODN but not with HVJ-liposome alone, sense, or scrambled ODN; (4) neointimal formation was accompanied by a significant decrease in vascular p53 protein as compared with HVJ-liposome alone or sense ODN; (5) the increase in neointimal area was abolished by cotransfection of wild-type p53 plasmid. Because p53 plays an important role in the regulation of apoptosis,\textsuperscript{6–8} decrease in apoptosis by antisense p53 ODN also might be involved in the neointimal formation. The present study is consistent with the previous report that the atherosclerotic lesions in p53\textsuperscript{−/−} /apoE\textsuperscript{−/−} mice had a significant increase in cell proliferation rate compared with those in p53\textsuperscript{+/+} /apoE\textsuperscript{−/−} mice.\textsuperscript{20} p53 may play a pivotal role in restenotic as well as atherosclerotic lesion development associated with its function in cell replication control.

To confirm the sustained effect of a single administration of ODN, we examined whether ODN persisted in the vessel wall by using the HVJ-liposome method. In previous studies, the HVJ-liposome–mediated transfer method for antisense ODN, which uses the fusion system mediated by HVJ, results in intracellular ODN delivery bypassing endocytosis.\textsuperscript{21,22} Consequently, this method increases the effectiveness and prolongs the half-life of antisense ODN in vitro.\textsuperscript{23} The present study demonstrates that the HVJ-liposome method actually prolongs the in vivo half-life of ODN. In fact, there is evidence that FITC-labeled ODN persist in the nucleus for at least 2 weeks after a single transfection. This method is clearly efficient for inducing sustained action of antisense ODN in vivo. Indeed, a high transfection efficiency of the HVJ-liposome method into blood vessels has also been reported.\textsuperscript{23} Yonemitsu et al\textsuperscript{23} documented that HVJ-liposomes could achieve highly efficient gene transfection into the medial smooth muscle cells of intact arteries at 150 and 760 mm Hg of pressure (mean=85.3\% and 93.5\% of total VSMC) without any inflammatory reaction for $\geq 14$ days.

Overall, the present study demonstrated that loss of p53 by antisense p53 ODN resulted in abnormal VSMC growth in vitro as well as in vivo. The observation in the present study is extremely important for understanding the molecular mechanisms of restenosis. In contrast, researchers have focused on the inhibitory effect of p53 on VSMC growth as a potential tool in molecular therapy to treat restenosis. Indeed,
overexpression of the p53 gene in the injured arterial wall inhibits the proliferation of VSMC in vivo. Alternatively, a single intraluminal incubation of human p21, identified as a downstream mediator of p53 gene, resulted in significant inhibition of neointimal formation after vein grafting, associated with a phenotypic change of VSMC from neonatal to adult type in a rabbit model. Further studies of p53 in the pathological conditions in vascular disease may provide new insights into therapeutic strategies.

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References


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