

# $\beta$ -Catenin, an Inducer of Uncontrolled Cell Proliferation and Migration in Malignancies, Is Localized in the Cytoplasm of Vascular Endothelium during Neovascularization after Myocardial Infarction

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**$\beta$ -catenin is a protein involved in cell-cell adhesion and proliferation. In neoplastic diseases, defects in the regulation of the cellular  $\beta$ -catenin content and cytoplasmic accumulation of the protein contribute to the uncontrolled cell proliferation and migration. Whether  $\beta$ -catenin plays a role in the controlled proliferative and migratory responses to injury, eg, of vascular endothelial cells during neovascularization after myocardial infarction (MI), is not known. In the present study, we examined the localization of  $\beta$ -catenin in the infarcted rat heart at different time points after MI. Cytoplasmic  $\beta$ -catenin was observed in the endothelial cells of the newly formed and pre-existing blood vessels in the infarct area in the first week after MI, but not in the uninjured parts of the heart and not at later time points. Adenomatous polyposis coli (APC) protein was also detected; interaction of APC with  $\beta$ -catenin has been reported to be critical in epithelial tube formation *in vitro*. Moreover, the expression of dishevelled-1, an upstream regulatory molecule of the cellular  $\beta$ -catenin content, was observed in vascular endothelial cells in the infarct area. These findings suggest a role for the  $\beta$ -catenin-APC complex in the proliferation and migration of vascular endothelial cells during neovascularization of the infarct area. (Am J Pathol 2000, 157:877–883)**

$\beta$ -catenin is an intracellular protein which can interact with different cellular targets. It was originally described as a component of adherens junctions. Recently, however, it has also been identified as an activator of gene expression. In cell adhesion, it functions in a complex of proteins that links cadherins, a family of transmembrane cell-cell adhesion receptors, to the actin cytoskeleton.<sup>1–3</sup> Modulation of gene expression by  $\beta$ -catenin is mediated

through its interaction with transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family in the nucleus.<sup>4–7</sup>

$\beta$ -catenin has recently received considerable attention in cancer research.<sup>8,9</sup> Elevated levels of  $\beta$ -catenin and a translocation of this protein to the cytoplasm have been associated with the development of colon carcinoma as well as a rapidly increasing number of other neoplastic diseases.<sup>4,6,10</sup> These elevated levels of  $\beta$ -catenin can be caused by mutations in the protein itself, but are more frequently the result of mutations in the adenomatous polyposis coli (APC) protein which regulates the degradation of  $\beta$ -catenin.<sup>11</sup> Mice lacking the  $\beta$ -catenin gene show lethal organizational defects early during development,<sup>12</sup> underscoring the crucial role for this protein in embryogenesis. This is further supported by studies which report a role for  $\beta$ -catenin during normal development.<sup>13–15</sup>

Taken together, the above-mentioned studies clearly define a role for  $\beta$ -catenin in the proliferative and migratory responses of cells during embryogenesis and in neoplastic disease. In contrast, little is known about a potential role for  $\beta$ -catenin in normal, controlled cell proliferation and migration during repair processes after injury, eg, of vascular endothelial cells during neovascularization of the infarct area after myocardial infarction (MI) in adult animals.<sup>16,17</sup> *In vitro* studies, however, have identified the cadherin-catenin complex as a crucial component of endothelial cell-cell junctions, which has to be dissociated and reorganized during angiogenesis.<sup>18</sup> In several reports, a decrease of membrane-bound and a concomitant increase in cytoplasmic  $\beta$ -catenin during the migration of human umbilical vein endothelial cells<sup>19,20</sup> and epithelial cells<sup>21</sup> has been described, underscoring the importance of the dissociation of the cadherin-catenin complex during angiogenesis.

The aim of the present study was to investigate the potential role of  $\beta$ -catenin in the neovascularization which

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occurs *in vivo* during infarct healing after MI. To this end, infarcted rat hearts were obtained at different time points (2 to 21 days) after MI and the  $\beta$ -catenin contents of the vascular endothelial cells around the infarct area and in the uninjured myocardium were compared. Because  $\beta$ -catenin-APC interactions have been shown to be critical for epithelial tubule formation *in vitro*,<sup>22</sup> we have performed immunohistochemical staining for APC as well. Moreover,  $\beta$ -catenin recently has been identified as a second messenger molecule in the Wnt polarity signal transduction pathway.<sup>15,23</sup> In this pathway, genes from the dishevelled family are known to regulate the degradation of  $\beta$ -catenin by controlling its phosphorylation by glycogen synthase kinase-3 $\beta$ .<sup>5</sup> Therefore we have studied the expression of the three murine dishevelled genes<sup>24–26</sup> in the infarcted rat heart by *in situ* hybridization.

## Materials and Methods

### Animals and Surgery

Adult male Wistar rats (250 to 300 g; Winkelmann, Borchen, Germany) were used in this study. They were housed in groups of 3 to 4 rats with free access to food and tap water. MI was induced as described previously.<sup>27</sup>

### In Situ Hybridization for Dishevelled-Homologues

The expression of the three dishevelled genes was studied by *in situ* hybridization. These experiments were performed on paraffin sections (4  $\mu$ m) from formalin-fixed infarcted rat hearts. The *in situ* hybridizations were performed as described previously.<sup>28</sup> Briefly, radiolabeled riboprobes were transcribed from polymerase chain reaction products of dvl-1, dvl-2, and dvl-3, obtained by amplification of reverse-transcribed RNA isolated from rat heart as previously described<sup>29</sup> (primer sets: dvl-1: upper CAGGGCACTGACAGCCAC, lower CAGTAGATGCACTGTCTGGAGG; dvl-2: upper AAGAGCGTTTTGCAGCGG, lower GACACAAGCCAGGAGACAAC; dvl-3: upper CCCCTTTCTGTGCTGACAAC, lower GCTCAATCCGGGAGACCTT), and cloned into a pGEM-T cloning vector (Promega, Madison, WI). The identity and orientation of the polymerase chain reaction products were verified by sequencing. RNA transcription was performed using an RNA labeling kit (Amersham, Little Chalfond, UK) in the presence of [<sup>35</sup>S]-UTP. The sections were hybridized overnight at 55°C with the radiolabeled probes. After washing the sections, unbound probe was digested with RNase (20  $\mu$ g/ml; Promega), the sections were dehydrated, dried, dipped in photographic emulsion (Kodak NTB2; Technorama, Zürich, Switzerland) and exposed for 1 to 2 weeks in the dark at 4°C. After development, the sections were briefly stained with hematoxylin.

### Immunohistochemistry

Immunohistochemistry was performed according to routine procedures. Paraffin sections were mounted on aminopropyltriethoxysilane-coated slides. A monoclonal antibody for  $\beta$ -catenin was obtained from Transduction Labs (Lexington, KY). After blocking the endogenous peroxidase, sections were boiled twice for 5 minutes in 10 mmol/L citrate buffer (pH 6.0) and incubated with the primary antibody in a 1:500 dilution overnight at room temperature. Immunohistochemistry for APC was performed using the N-15 monoclonal antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA) in a 1:2,000 dilution and incubating for 1 hour at room temperature. Sections were pretreated with 1 mg/ml of pepsin (Boehringer, Mannheim, Germany). Biotinylated multilink swine-anti-goat, -mouse, and -rabbit secondary antibody (dilution 1:100; DAKO, Glostrup, Denmark) and the Vectastain ABC kit (Vector, Burlingame CA) were used according to the manufacturers' instructions to visualize the binding of both primary antibodies. Sections were briefly counterstained with hematoxylin and mounted with Entellan (Merck, Darmstadt, Germany). The identity of vascular smooth muscle cells and myofibroblasts was determined using an antibody against  $\alpha$ -smooth muscle actin (dilution 1:500; DAKO).

### Confocal Microscopy

Confocal microscopy was performed using a Bio-Rad MRC600 confocal scanning laser microscope (Bio-Rad, Hempel Hemstead, UK) as previously described.<sup>30</sup> Immunohistochemistry for  $\beta$ -catenin was performed as described above with fluorescein isothiocyanate-labeled rat-anti-mouse (dilution 1:100; DAKO) as secondary antibody. The nuclei were counterstained using propidium iodide.

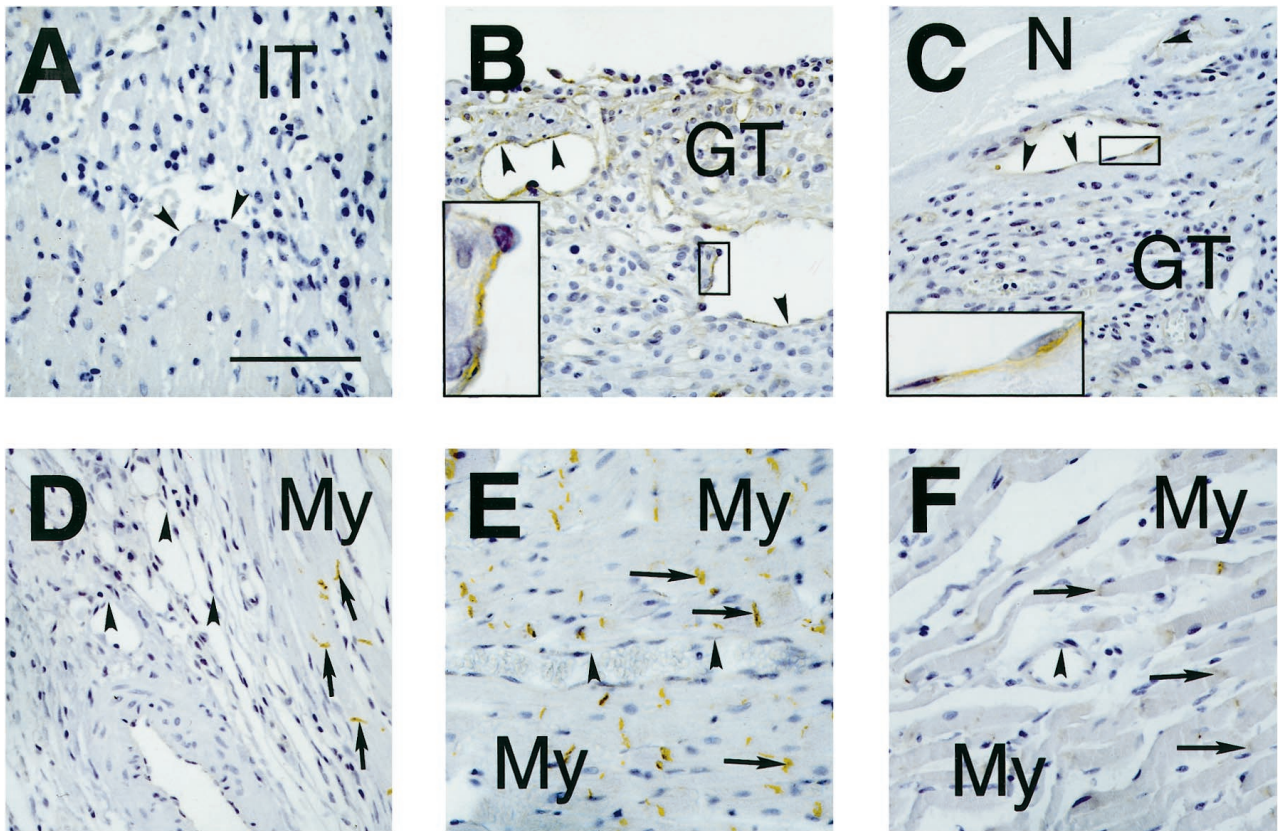
### BrdU Incorporation

BrdU (Serva, Heidelberg, Germany) was continuously infused using osmotic minipumps (Alzet model 2002; Alza Corp., Palo Alto, CA) implanted subcutaneously between the shoulder blades. BrdU was dissolved in 0.9% NaCl (10 mg/ml) and infused at a rate of 240 ng/kg/min. The pumps were implanted at the time of MI induction and animals were sacrificed 4 and 7 days after MI. The cumulative labeling of BrdU in the infarct area was determined using a monoclonal antibody directed against BrdU according to previously published methods.<sup>31</sup>

## Results

### Time Course of the $\beta$ -Catenin Expression in Newly Formed Vessels around the Infarct Area

The expression of  $\beta$ -catenin in the infarcted part of the left ventricle of the rat at different time points after MI is



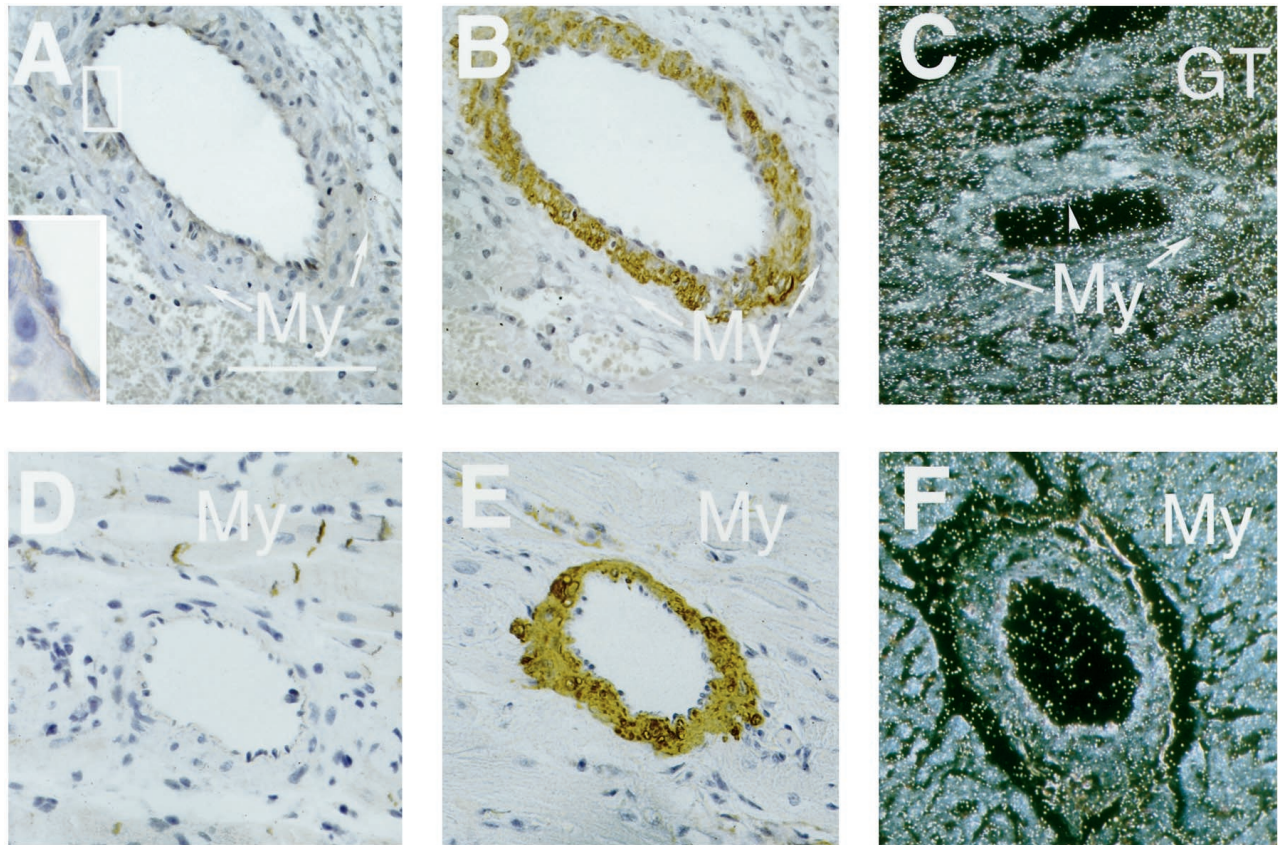
**Figure 1.** Time course of cytoplasmic  $\beta$ -catenin localization in small blood vessels in the area of infarction in sections of infarcted rat hearts. **Arrowheads** indicate the vascular endothelial cell layer whereas **arrows** indicate the intercalated disks of cardiomyocytes. **A:** Two days after MI neovascularization has not yet started. The small vessels in the infarct area do not show  $\beta$ -catenin staining, probably because these vessels are remnants of the vasculature present before infarction. The border zone of the infarct consists of inflammatory tissue (IT). Scale bar, 100  $\mu$ m. **B:** Four days after MI many newly formed small vessels were present in the border zone of the infarct area. In the cytoplasm of the endothelial cells  $\beta$ -catenin staining was observed (see high-power magnification in **inset**). In contrast, the granulation tissue (GT), which is formed in the border zone of the infarct, did not show detectable  $\beta$ -catenin staining. **C:** Seven days after MI, the cytoplasm of the vascular endothelial cells of small vessels in the infarct area was still positively staining for  $\beta$ -catenin (**inset** shows high-power magnification). The granulation tissue again was negative. **D:** Twenty-one days after MI, no  $\beta$ -catenin staining could be detected in most of the vascular endothelial cells of the small vessels. The intercalated disks of the cardiomyocytes (My) stained positively for  $\beta$ -catenin. **E:** A small vessel in a noninfarcted area of the heart, 4 days after MI, is shown. No  $\beta$ -catenin staining of the cytoplasm of the vascular endothelial cells could be observed. The intercalated disks of the myocytes were positively staining for  $\beta$ -catenin. **F:** Small vessel in a noninfarcted part of the heart, 7 days after MI. Again no positive staining of vascular endothelial cells was observed.

shown in Figure 1. Two days after infarction, no staining for  $\beta$ -catenin was observed around the area of infarction (Figure 1A). In this phase of the wound healing no neovascularization or formation of granulation tissue has yet occurred in the infarct area. At 4 days after MI, however, many small blood vessels have emerged in the border zone around the area of infarction. Interestingly, the cytoplasm of the vascular endothelial cells stained positively for  $\beta$ -catenin (Figure 1B). This staining was not observed in vascular endothelial cells in the noninjured myocardium (Figure 1E) and in vascular endothelial cells of sham-operated animals (not shown).  $\beta$ -catenin staining of vascular endothelial cells was also observed 7 days after infarction (Figure 1C). In contrast, the myofibroblasts present in the granulation tissue around the area of infarction did not show any  $\beta$ -catenin staining, and vascular endothelial cells in noninfarcted parts did not show cytoplasmic  $\beta$ -catenin staining (Figure 1F). At 14 (not shown), 21 (Figure 1D), and 90 days (not shown) after MI, most of the vascular endothelial cells in the areas of newly formed blood vessels around the infarct

did not show  $\beta$ -catenin staining. Again, the myofibroblasts did not show any staining for  $\beta$ -catenin. The intercalated disks between the uninjured cardiomyocytes were positive for  $\beta$ -catenin (Figure 1, D–F) at all time points as previously reported.<sup>32</sup>

#### *$\beta$ -Catenin Expression in Larger, Muscular Arteries in the Infarct Area*

Cytoplasmic  $\beta$ -catenin staining of the vascular endothelium was not confined to the newly formed small vessels around the infarct area, but could also be observed in larger arteries in the injured parts of the heart (Figure 2A). These arteries most likely were already present in the area before the infarction, because a fully developed media containing vascular smooth muscle cells (Figure 2B) and a layer of surviving cardiomyocytes around these arteries was observed around them. In contrast, arteries of similar diameter in the uninjured parts of the heart



**Figure 2.** **A:**  $\beta$ -catenin staining of endothelial cells of a larger, pre-existing artery in the area of infarction, 4 days after MI. Around the artery a thin layer of surviving cardiomyocytes (My) was present, confirming that the artery was present before infarction. Scale bar, 100  $\mu$ m. **B:** Immunohistochemistry for  $\alpha$ -smooth muscle actin (ASMA) shows staining of the vascular smooth muscle cells in the media of the same artery, but not of the endothelial cell layer or the cardiomyocytes. **C:** Dark-field image of an *in situ* hybridization with a dishevelled-1 (dvl-1) probe of a section of rat heart, 4 days after MI. The photographic grains are represented as white spots. Note that grains are localized over the vascular endothelium (**arrowhead**), whereas the amount of grains over the cardiomyocytes (My) is lower. A high density of grains was observed over the granulation tissue (GT) in the border zone of the infarct. **D** and **E:** Muscular arteries in the noninfarcted parts of the myocardium did not show endothelial  $\beta$ -catenin staining, but the vascular smooth muscle cells stained positively for (ASMA). **F:** No dvl-1 expression was observed in muscular arteries in the noninfarcted areas of the heart.

(Figure 2, D and E) or in sham-operated animals (not shown) did not show endothelial  $\beta$ -catenin staining.

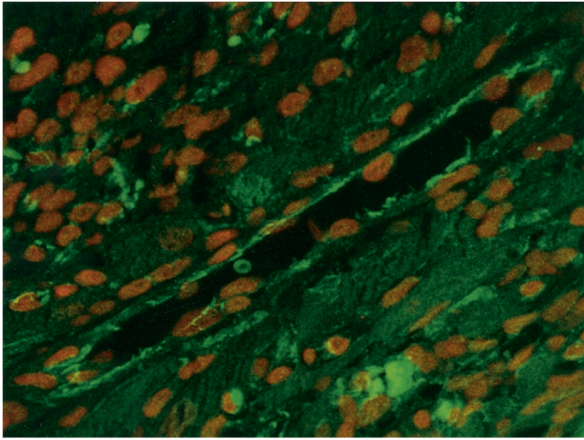
Because  $\beta$ -catenin is known to act as a second messenger in the Wnt-frizzled-dishevelled signal transduction pathway, we performed *in situ* hybridization for the three known murine dishevelled (dvl) homologues. As shown in Figure 2C, silver grains were observed over the endothelial cells of the larger arteries in the infarct area when the dvl-1 probe was used. In contrast, labeling above background levels could not be detected in these cells using the dvl-2 or the dvl-3 probe (not shown). Muscular arteries in the uninfarcted parts of the heart did not show signal above background levels with the dvl-1 probe (Figure 2F). Abundant labeling with the dvl-1 probe was also observed in the granulation tissue around the infarct area, which is rich in (myo)fibroblasts and monocytes (Figure 2C). This abundant labeling obscured the unambiguous detection of dvl-1 signal in endothelial cells of the small, newly formed vessels, although in the dvl-1 autoradiograms the density of silver grains in areas with many newly formed blood vessels was above background levels.

### Confocal Microscopy of $\beta$ -Catenin

To determine the intracellular localization of  $\beta$ -catenin in more detail, confocal microscopy was performed on sections of infarcted rat heart 4 and 7 days after MI. As shown in Figure 3, immunofluorescence was observed in the cytoplasm of the vascular endothelial cells around the infarct area but not in the nuclei, as observed with immunohistochemistry.

### Localization of APC Protein in the Infarcted Rat Heart

The expression of the APC protein, involved in the degradation of  $\beta$ -catenin, was studied in infarcted rat tissue after MI. Seven days after MI, some staining for APC protein was observed in the endothelial cells of the newly formed vessels around the infarct area (Figure 4A). A similar pattern of staining, although of considerably higher intensity, was observed 21 days after MI (Figure 4B), and positive vascular endothelial cells could still be

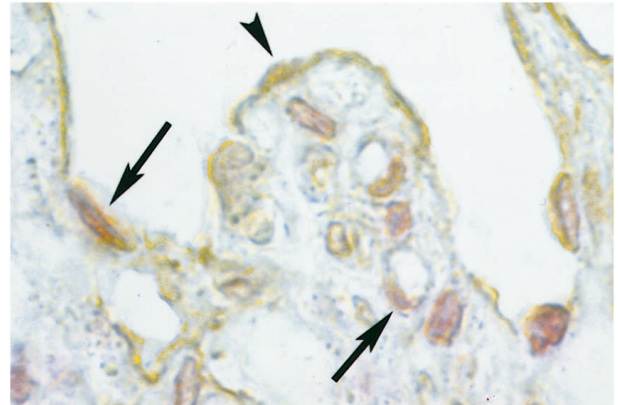


**Figure 3.** Confocal microscopy of a small vessel in the infarct area of a rat heart, 7 days after MI.  $\beta$ -Catenin staining is depicted in green and the nuclear counterstain (propidium iodide) is depicted in red. Note that the  $\beta$ -catenin staining is present in the cytoplasm of the vascular endothelial cells, whereas  $\beta$ -catenin staining in the nuclei could not be detected.

detected at 90 days after MI (not shown). Surviving cardiomyocytes close to the area of infarction showed a diffuse staining for APC protein (Figure 4A), which was not observed in cardiomyocytes in the uninjured parts of the heart. APC staining was also observed in vascular smooth muscle cells at all time points studied.

#### Double-Staining for $\beta$ -Catenin and BrdU

Because cytosolic  $\beta$ -catenin accumulation has been associated with cell proliferation in a number of neoplastic diseases, we performed immunohistochemical double-staining for  $\beta$ -catenin and BrdU, a nucleotide analogue which is incorporated in the DNA during cell proliferation. As shown in Figure 5, positive nuclei were observed in

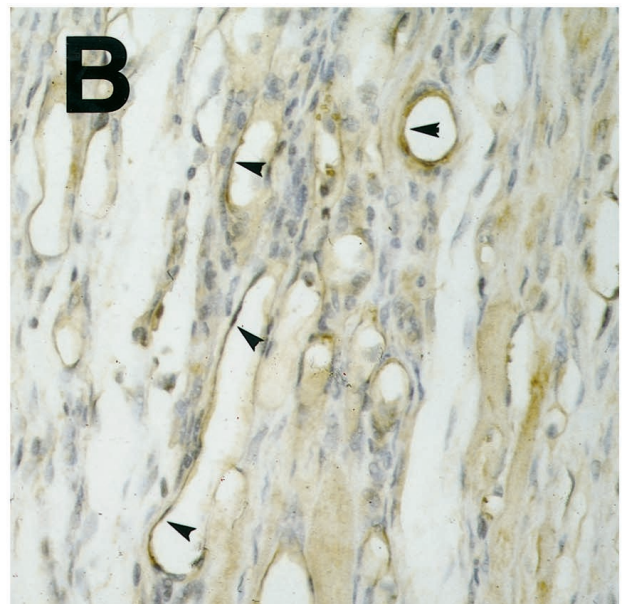
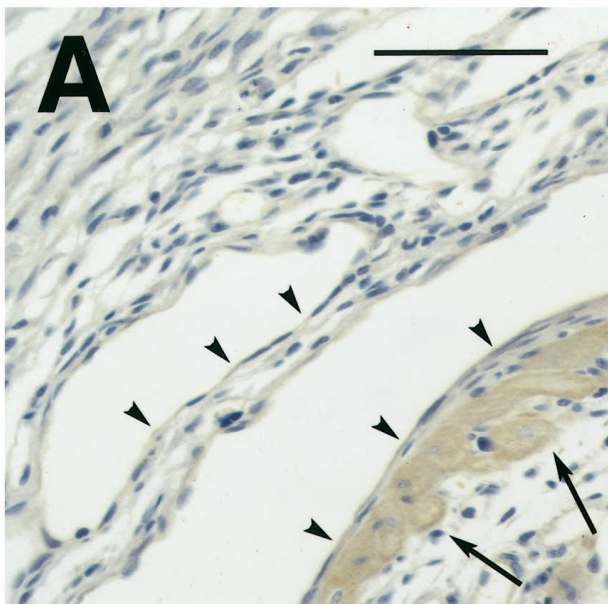


**Figure 5.** High-power magnification of immunohistochemistry for BrdU and  $\beta$ -catenin in a small vessel and capillaries in the infarct area of a rat heart, 4 days after MI. BrdU-positive nuclei of proliferating cells are depicted in red, and  $\beta$ -catenin is shown in brown. Some of the vascular endothelial cells that contain  $\beta$ -catenin in their cytoplasm have a BrdU-positive nucleus (**arrows**), but  $\beta$ -catenin-positive cells without nuclear BrdU incorporation were also observed (**arrowhead**).

some, but not all of the vascular endothelial cells that stained positive for  $\beta$ -catenin. Because BrdU incorporation was not a generalized phenomenon in  $\beta$ -catenin-positive vascular endothelial cells, this suggests that the cytoplasmic  $\beta$ -catenin in these cells is not a direct inducer of cell proliferation.

#### Discussion

The aim of the present study was to disclose a potential link between the presence of  $\beta$ -catenin in the cytoplasm of the vascular endothelial cells and their proliferation and migration during the process of neovascularization in infarct healing. To this end, we have studied the localiza-



**Figure 4.** Localization of the APC protein in vascular endothelium during infarct healing in the rat. APC staining was observed in the endothelial cells of newly formed vessels around the infarct area at 7 days after MI (**A**). In contrast to the  $\beta$ -catenin staining, APC staining was still observed in these cells 21 days after MI (**B**).

tion of  $\beta$ -catenin, its regulatory protein APC, and the expression of the dishevelled genes, located upstream in the Wnt-dishevelled- $\beta$ -catenin signal transduction pathway, in infarcted rat hearts at different time points. Because the neovascularization which takes place after MI consists of angiogenesis as well as the remodeling of pre-existing arteries,<sup>16</sup> processes which are both dependent on endothelial cell proliferation and migration,<sup>17</sup> this model seems to be very suitable to study a link between  $\beta$ -catenin expression and the proliferation and migration of endothelial cells *in vivo*.

$\beta$ -catenin was observed in the cytoplasm of the endothelial cells of newly formed vessels around the area of infarction 4 and 7 days after MI. In contrast, vascular endothelial cells in the noninfarcted part of the heart showed no detectable  $\beta$ -catenin levels in their cytoplasm. Because remodeling of existing capillaries as well as *de novo* generation of capillaries take place in the first week after MI,<sup>17,33,34</sup> the time frame of the appearance of cytoplasmic  $\beta$ -catenin matches with that of the neovascularization. Based on *in vitro* data in which the appearance of  $\beta$ -catenin in the cytoplasm was observed during human umbilical vein endothelial cell migration,<sup>19,20</sup> our observations suggest a similar translocation phenomenon in endothelial cell proliferation and migration *in vivo*. These observations are also in accordance with *in vitro* studies in which the vascular endothelium (VE)-cadherin-catenin complex was found to be essential for *de novo* capillary formation<sup>35</sup> and in which vascular endothelial growth factor-induced phosphorylation of the cadherin-catenin complex was found to be essential for the loosening of endothelial cell-cell contacts, a first step in angiogenesis.<sup>36</sup> Moreover, in a recent study by Carmeliet et al<sup>37</sup> it was shown that the VE-cadherin/ $\beta$ -catenin complex is essential for endothelial cell remodeling and maturation during gestation in mice, and that targeted disruption of the intracellular part of the VE-cadherin gene leads to abnormal vascular development, underscoring the importance of these molecules in the formation and remodeling of blood vessels. Moreover,  $\beta$ -catenin translocation to the cytoplasm was observed in endothelial cells in culture during their remodeling in response to shear stress, suggesting that this is likely to be a normal phenomenon during rearrangement of endothelial cells.

An abundant presence of APC protein was observed in vascular endothelium and myocytes around the infarct area as well as in vascular smooth muscle cells. In a recent study by Pollack et al,<sup>22</sup> a functional interaction between  $\beta$ -catenin and APC has been shown to be necessary for tubule formation of Madin Darby canine kidney cells *in vitro*. Moreover, there is increasing evidence that APC itself is involved in the formation of stable membrane extrusions during active cell migration.<sup>38</sup> It is tempting to speculate that the co-expression of  $\beta$ -catenin and APC in the area of neovascularization around the infarct area promotes vascular endothelial cell proliferation and migration in both angiogenesis and remodeling of pre-existing vessels. On the other hand, the rise in APC protein content which coincides with the decrease in  $\beta$ -catenin content of the vascular endothelial cells may suggest that the APC protein contributes to the  $\beta$ -catenin degradation

in these cells. The role for APC in vascular smooth muscle cells and cardiomyocytes, however, is unclear.

Recently,  $\beta$ -catenin has been identified as a second messenger in the signal transduction cascade of Wnt proteins, which are involved in control of tissue polarity.<sup>15,23</sup> In the original description of this signal transduction system in *Drosophila*, the dishevelled protein was shown to act directly downstream of frizzled, now recognized as the receptor for proteins from the Wnt family.<sup>39</sup> In the mean time, three mammalian dishevelled homologues have been identified in the mouse.<sup>24–26</sup> Using these mouse sequences, we have designed primer sets which allowed polymerase chain reaction-amplification of three fragments from rat DNA. Partial sequencing of these fragments revealed homologies of >90% with the published mouse sequences, confirming the identity of these fragments as rat homologues of mouse dishevelled 1–3. The three polymerase chain reaction fragments were subsequently used for *in situ* hybridization. In sections of infarcted rat hearts, 7 days after MI, expression of *dvl-1* was detected in vascular endothelial cells of the larger vessels around the infarct area, but not in uninfarcted areas of the heart. Areas rich in newly formed capillaries around the infarct area also showed signal for *dvl-1* above background levels, but because the resolution of the radioactive *in situ* technique is not high enough to allow precise cellular localization, the interpretation of these data is difficult. This problem is further aggravated by the high levels of *dvl-1* expression in the myofibroblasts, which are abundant around the area of infarction.<sup>28,33</sup> However, from the data obtained with the larger arteries we can conclude that a key upstream component of the Wnt-frizzled-dishevelled tissue polarity cascade is present in the vascular endothelial cells during neovascularization of the infarct area, which may suggest a role for this pathway in the control of proliferation and/or migration of these cells. This concept is supported by a recent study of Dupl aa et al<sup>40</sup> in which a relation between the level of expression of FrzA, a scavenger for Wnt proteins which is structurally related to frizzled receptors, and endothelial cell proliferation *in vitro* was demonstrated. Moreover, recently the presence of a Wnt/frizzled signal transduction system which, when activated, increases the cytoplasmic  $\beta$ -catenin content has been described in vascular endothelial cells in primary culture,<sup>41</sup> providing additional evidence that Wnt/frizzled activation may be involved in the  $\beta$ -catenin translocation observed in the present study.

In conclusion, the present study shows the presence of cytoplasmic  $\beta$ -catenin and APC protein in vascular endothelial cells during neovascularization after MI. Because  $\beta$ -catenin is known to be a key regulator of both cell proliferation and migration, a role for this protein in angiogenesis and vascular remodeling can be anticipated. This is further substantiated by data from *in vitro* studies in which translocation of  $\beta$ -catenin to the cytoplasm has been linked to angiogenesis. Moreover, the expression of *dvl-1* in these cells suggests an involvement of the Wnt-dishevelled- $\beta$ -catenin polarity cascade in this process. These findings underscore that  $\beta$ -catenin is not only a crucial factor during development and in

neoplastic disease, but can also play a role in the vascular endothelial cell proliferation and migration during neovascularization after MI.

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