DNA Synthesis and Mitoses in Coronary Collateral Vessels of the Dog

By Wolfgang Schaper, Marc De Brabander, and Paul Lewi

ABSTRACT

DNA synthesis and mitotic activity in coronary collateral arterioles was assessed in dogs at different time intervals after gradual ameroid constriction of the left circumflex coronary artery. Labeling of nuclei and the mitotic index were highest at 3 weeks after implantation of the constrictor and gradually declined thereafter. Labeling persisted for at least 8 weeks but radioactive DNA was not found 12 months after coronary artery constriction nor in controls or animals with sham operations. Proliferative activity was, at any time interval, highest at the level of the smallest diameters of the collateral vessels. Labeled nuclei and mitoses were found in endothelial, medial, and adventitial cells. Myocardial mesenchymal cells also incorporated tritiated thymidine. The data provide evidence that after constriction of a major coronary artery, the coronary collateral vessels enlarge by an active growth process that follows the basic laws of cell kinetics.

KEY WORDS autoradiography DNA synthesis ischemia cell division coronary collateral circulation ameroid constriction vessel wall growth

Myocardial ischemia leads to an enlargement of collateral vessels in man (1-5) as well as in animals (6-10). However, uncertainties exist as to the mechanism of this increase in vascular caliber. Early but scanty observations (11) showed that the collateral vessels are overstretched arterioles "lacking an arterial coat" and state that these vessels are preexistent and open up under the influence of pressure gradients and metabolic and neurogenic factors (12). However, several reports from our laboratory (7,13-21) have suggested that collaterals grow actively rather than expand passively when the surrounding cardiac muscle is made ischemic. These observations were based on the chance finding of smooth muscle cells in mitosis and on the fact that the volume of the vascular wall increases (14, 17).

The final and formal evidence of arterial growth in the presence of myocardial ischemia was, however, never established. The material presented in this study provides evidence that DNA synthesis and mitoses are responsible for the growth and expansion of collateral vessels. This was achieved by the detection of radioactive DNA in enlarged collaterals of dogs with chronic coronary artery occlusion after injection of a labeled precursor of DNA, tritiated thymidine. Furthermore, the determination of the labeling index as a function of time after experimental coronary artery occlusion made possible an estimate of the speed with which arterial smooth muscle cells are able to reproduce.

Method

Details of the surgical procedure have been described elsewhere (17). In brief, ameroid constrictors (22) were implanted around the circumflex branch of the left coronary artery of randomly selected mongrel dogs of either sex with an average weight of about 18 kg. The ameroid plastic is a hygroscopic material which swells with uptake of tissue fluid. Since the material is encased by a stainless steel ring, it constricts the coronary artery over a period of 28 weeks (7).

After the implantation, which was carried out under anesthesia and artificial respiration using clean surgical techniques, the animals were allowed to recover. Three, 4, 8, and 52 weeks after the operation, groups of dogs were again
Arteriographic demonstration of a large coronary collateral bypassing a chronic coronary artery occlusion. The collateral is divided into stem (S), midzone (M), and reentry (R) by the heavy bars. Fine and heavy bars indicate the borders of vascular tissue blocks. The plane of sectioning is always at the reentry end of the blocks. LAD = left anterior descending coronary artery; LCCA = left circumflex coronary artery; AC = constrictor.

Anesthetized, the chest was opened under artificial respiration and a heart-lung preparation was set up. Three millicuries of tritiated thymidine were injected into the venous reservoir and the heart-lung preparation was continued for approximately 3 hours. Then the venous reservoir was emptied and the heart was perfused with oxygenated Tyrode’s solution to remove as much blood and free radioactivity as possible. The heart was then fixed by perfusion with 4% phosphate-buffered formaldehyde, rinsed again with Tyrode’s solution to remove the non-tissue-bound formaldehyde, and a suspension of micronized barium sulfate in a 9% gelatin solution was infused into the coronary arteries via the aorta under a pressure of 100 mm Hg. The white barium mass made enlarged collaterals clearly visible to the naked eye, but arteriograms were also taken in order not to miss endomural collaterals (Fig. 1). All identifiable collaterals were excised over their entire length from stem to reentry into the recipient artery.

According to Longland’s definition (23) the “stem” is a subbranch of a coronary artery from which the collateral artery originates. The “midzone” is the part of the collateral located between stem and reentry and is expected to show the most obvious and dramatic growth transformation because of its very small initial diameter (40 μm on the average). The reentry is a subbranch of the occluded coronary artery. These collaterals were cut perpendicular to their longitudinal axis and divided into small blocks of about 5 mm. Paraffin sections of about 6 μm were cut and stained with PAS and hematoxylin-eosin. The sections prestained with PAS were coated in the darkroom with liquid nuclear emulsion (Ilford L4) and stored in light-tight containers according to Schaper and Van Even (24) for 4 weeks at 4°C. After exposure, the slides were developed for 3 minutes in Kodak D19 developer, fixed, washed and counterstained with Harris-hematoxylin.

Radioactive DNA was made demonstrable by reflected light microscopy according to Rogers (25) with the Leitz Ultropak System.

In six animals, demecolcine (Colcemid) in a dose of 0.5 mg/kg was injected intravenously approximately 3 hours before the heart-lung preparation was started.

In addition to the 11 animals with chronic coronary artery occlusion, 3 normal dogs and 3 dogs with sham operations were used as controls. In dogs with sham operations, the same surgical procedure was followed with the exception of the constrictor implantation. Three weeks after the sham operation, a heart-lung preparation was set up as described above, and the same amount of tritiated thymidine was added to the venous reservoir. Identical heart-lung preparations were made in the three normal dogs. In the normal dogs and in the dogs with sham operations, vessels comparable in size with enlarged collaterals (between 400 and 1600 μm) and vessels from the nonenlarged arteriolar collateral network were excised and processed as described above. In addition to the two types of control experiments, normal vessels (i.e., unrelated to collaterals) from nonoccluded coronary arteries were taken from the hearts with chronic coronary artery occlusions. These vessels were also processed for autoradiography.

Determination of the Labeling Index.—Labeled nuclei are easily identifiable in emulsion-covered histological sections with reflected light microscopy (Fig. 2). All labeled nuclei in each

Obtained from the Radiochemical Centre, Amersham, U. K.

Ciba AG, Switzerland.
FIGURE 2

Autoradiographic demonstration of radioactive DNA in nuclei of coronary vascular smooth muscle cells in part of a collateral vessel studied 3 weeks after constrictor implantation. Clusters of developed silver grains (bright white) indicate the presence of radioactive DNA in 3 nuclei of smooth muscle cells. The tissue was stained with periodic acid-Schiff and counterstained with Harris-hematoxalin. The section was photographed with transmitted light (tissue) and with reflected light (silver grains) illumination. The lumen (L) is filled with micronized barium sulfate-gelatin.

Results

Labeling Index as a Function of Time.---

The largest number of labeled nuclei was found in the group of animals 3 weeks after constrictor implantation (Figs. 4 and 5). In animals studied at later sampling intervals (4 and 8 weeks after the operation) labeling had declined.

Parallel with the decrease in the number of DNA-synthesizing cells, the total cell population of the collaterals rose from an initial value of about 30 cells/6 μm tissue section to about 300 cells 8 weeks after constrictor implantation (Fig. 6). The decline in the number of DNA-synthesizing nuclei is statistically significant (P = 0.0040) when the dogs studied 3 weeks after constriction are compared with those studied 8 weeks after constriction. Labeled nuclei could not be detected in dogs studied 1 year after the operation, nor were they found in normal dogs or in dogs with sham operations. Labeled nuclei were also lacking in the normal coronary vessels of the...
animals with chronic coronary artery occlusion.

The percent of labeled nuclei over the entire length of the excised collaterals at the 3-week sampling interval was 3.5% of all endothelial and medial cells. The percent of labeled midzone nuclei was 5.5%, indicating that the frequency of DNA-synthesizing nuclei falls off toward both ends of the midzone. The highest frequency of labeling ever observed in a 3-week vascular segment was 8%, an average of 20 serial sections taken in sequence from a midzone block. The highest absolute number of labeled nuclei in a single 6 μm section of a collateral midzone was 43 labeled nuclei in a vessel wall with an internal diameter of 310 μm.

Labeled nuclei were also found in the adventitia and in the myocardium surrounding the growing vessels. These cells could easily be identified as fibroblasts when found in the adventitial space or as mesenchymal myocardial cells. However, labeled cardiac muscle cells could not be identified with certainty. Occasionally it was possible to identify radioactive DNA in the nuclei of myocardial capillaries. In these cases, identification was aided by the "buckshot" appearance of the perfusion-fixed tissue: all capillaries were fixed in the widely patent state.

Frequency of Mitoses as a Function of Time.—Six animals received demecolcine, which stops mitoses in the metaphase. Only nuclei in the metaphase were counted in hematoxylin-eosin stained sections or in sections with Weigert's hematoxylin-elastica van Gieson. Although the time during which demecolcine could halt mitoses was about 6 hours, the number of counted mitoses was surprisingly low. At the 3-week sampling, 23 mitoses were found in 22 sections (average number of counted sections per heart), i.e., roughly 1 mitosis/section of 6 μm.

At the 8-week sampling, the number of mitoses decreased to 8/22 sections. There were no mitoses in normal dogs or in dogs with sham operations. Mitotic activity had
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Number of labeled nuclei and labeling index as a function of time after implantation of the constrictor. Total amount of vascular label (endothelial plus medial plus adventitial labeled nuclei) per heart, which is the average per 24.5 vascular sections (Table 1). Each point represents one heart. Note the decrease in labeling as a function of time after operation. The difference of the means between 3 and 8 weeks has a random probability of 0.004.

also ceased completely in dogs with chronic 1-year coronary artery occlusion.

Most of the mitoses were found in the tunica intima and in the tunica media of the collaterals. Often it was not possible to classify these mitoses into endothelial or smooth muscle cells because of the dedifferentiated state that is a normal phenomenon during this stage of cellular reproduction.

Discussion

It is difficult to draw quantitative conclusions from histological material and even more difficult to test these results statistically. A tissue section is only a sample that the investigator hopes is representative of at least the block of tissue from which it was taken. When comparing coronary vessels from different animals and at different time intervals after coronary artery occlusion, one must be certain that a comparable number of vessels is studied, that a comparable number of blocks is taken from each heart, and that a comparable number of sections is prepared from these blocks. Table 1 shows that sampling errors have not been made.

The reliability of the tritiated thymidine technique as a measure of cell proliferation has been sufficiently established elsewhere (27). Furthermore, the observed increase in total cell number, together with the occurrence of mitosis, indicates that the incorporation of radioactive thymidine is due to reduplication of DNA in preparation for nuclear division and not to metabolic DNA turnover (28).

Several studies have failed to show uptake of tritiated thymidine or mitotic activity by cells of the normal vascular wall (29, 30).

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Increase in the total population of endothelial and smooth muscle nuclei per midzone section as a function of time. The total cell population rises from a normal value of 30 (=midzones of normal hearts, obtained from ±40% vessels) to a final value of 300. The difference of the means between 3 and 8 weeks has a random probability of 0.0000012.

Labeling of endothelial cells, which have to withstand the shear forces of the pulsatile pressure and flow, was detected in the guinea pig by Wright (31) after infusion of tritiated thymidine. The only study that succeeded in demonstrating labeled cells throughout the vascular tree of normal adult animals was that of Spaet and Leijnicks (32). However, the authors stated that the animals, laboratory rabbits, continue to gain weight as they age, even after complete maturity. Hence, the vascular bed is also growing.

In our control experiments, labeled cells were never found in normal coronary arteries and arterioles after 3 to 4 hours of exposure to tritiated thymidine in a heart-lung preparation. Hence the cells of the vascular wall in normal adult animals should be classified as a stable or normally nongrowing cell population (33).

On the basis of labeling studies with tritiated thymidine in rats at 3 days and 6 months after birth, Leblond (33) arrived at an experimental classification of cell population types. Cell populations which had retained for 6 months most of the label received at 3 days but showed no new labeling when given an injection of tritiated thymidine 6 months after birth were called the stable or nongrowing cell populations. Striated, smooth, and cardiac muscle and the intermediate-sized neurons of the brain belong to these populations. Cell populations labeled at 3 days as well as at 6 months were called expanding cell populations. Cell populations which took up tritiated thymidine at both intervals but showed no label 6 months after injection received at 3 days were called renewing cell populations.

When we apply this classification to our model of vascular growth, we reach the following conclusion. Under the influence of chemical (hypoxia) and physical (13) factors, a stable or nongrowing cell population (vascular smooth muscle) suddenly adopts the behavior of an expanding cell population—the vessel grows larger and the number of cells increases. In the absence of stimulation (diminished tissue hypoxia due to collateral growth) the expanding cell population reverts to a stable nongrowing cell population. It is quite obvious that the system shows the features of a self-controlling growth process (34). As with partial hepatectomy and other experimental models of this type, there is an initial fast mitotic response to a certain stimulus that quickly reaches a maximum and is followed by a gradual decline toward normal activity as the size of the population reaches critical values.

In our model, however, neither the exact onset of cell division and the moment of peak activity nor the exact time when cell turnover has reached its normal value again is known because these points were not included in the

**TABLE 1**

<table>
<thead>
<tr>
<th>Analysis of Possible Sampling Errors</th>
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<tbody>
<tr>
<td>3 wk</td>
</tr>
<tr>
<td>Collaterals/heart</td>
</tr>
<tr>
<td>Blocks/heart</td>
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<tr>
<td>Vascular sections/heart</td>
</tr>
</tbody>
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*Number of pieces too small for comparison.

The number of collaterals, blocks, and vascular sections per heart were not significantly different from each other in the 3, 4, and 8 week periods.

**FIGURE 6**

Increase in the total population of endothelial and smooth muscle nuclei per midzone section as a function of time. The total cell population rises from a normal value of 30 (=midzones of normal hearts, obtained from ±40% vessels) to a final value of 300. The difference of the means between 3 and 8 weeks has a random probability of 0.0000012.
observation period. Most probably, cell division started before 3 weeks after implantation of the constrictor. Indeed, occlusion of the circumflex artery has been shown statistically (7) to occur at 2/2 weeks after the operation. Because this occlusion is most probably the trigger that induces collateral growth, maximal stimulation should be expected at that moment. It seems likely, moreover, that even before total occlusion, when the stenosis has reached a critical value, cell growth has already started.

Vascular DNA synthesis and subsequent mitosis lead to an expansion of the initial cell population by a factor of approximately 10 (Fig. 6). This increase is due solely to mitosis (details of the calculations leading to this conclusion are presented in the Appendix).

The agreement between the percent of labeled nuclei and the increase in the cell population is sufficient reason to accept the hypothesis that enlargement of collaterals is the result of active growth rather than passive stretch. The relatively long duration of the S phase (the time necessary to duplicate DNA) of the proliferative cycle of the arterial smooth muscle cells (22 hours, see Appendix) explains why the collateral circulation cannot prevent myocardial infarction when a large coronary artery is suddenly occluded. However, when such an occlusion develops more slowly, a sizeable amount of cardiac muscle can be saved through growth transformation of the collateral circulation. Autopsy findings have shown that this can also occur in the human heart (8).

Only speculations can be offered at present as to which biochemical agents and which physical forces initiated vascular growth. We believe that the mechanisms which are responsible for coronary autoregulation (35) may play a role whenever the dilative stimulus reaches a certain intensity and lasts for a sufficiently long time.

Appendix

Because the cells of the vascular tree are generally believed to form a stable nongrowing population and hence normally do not synthesize DNA, it seemed of interest to estimate the length of the S phase of the cell division cycle in a case in which these cells do form new DNA.

Let \( N(t) \) be the total number of cells at time \( t \), \( f(t) \) the percent of cells with labeled nuclei at time \( t \), \( t \) the duration of phase S in hours, and \( s(t) \) the rate of the transition of cells into phase S at time \( t \) (hr\(^{-1}\)). Then the percent of cells in phase S at any time \( t \) is given approximately by

\[
s(t) \cdot t \cdot 100,
\]

provided \( s(t) \) does not change significantly over the period \( t \).

When \(^3\)H-thymidine is administered for 3 hours, the percent of labeled cells will be defined by

\[
f(t) = s(t) \cdot (t + 3) \cdot 100.
\]

The rate of increase of cells can be computed from

\[
dN = s(t) \cdot N(t) \cdot dt \frac{f(t)}{(t + 3) \cdot 100} \cdot N(t) \cdot dt
\]

and

\[
\frac{dN}{N(t)} = 0.01 \frac{f(t) \cdot dt}{t + 3}.
\]

By integration we obtain

\[
\ln \frac{N(t)}{N(t_0)} = 0.01 \int_0^t f(t) \cdot dt.
\]

If \( S(t_0,t) \) denotes the area under the curve of percent labeled nuclei versus weeks after implantation, we may substitute

\[
\int_0^t f(t) \cdot dt = (24 \times 7) \cdot S(t_0,t)
\]

\[
= 168 \cdot S(t_0,t).
\]

Hence

\[
\ln \frac{N(t)}{N(t_0)} = 1.68 \frac{S(t_0,t)}{t + 3},
\]

from which we can derive

\[
t = \frac{1.68}{\ln \frac{N(t)}{N(t_0)}} (S(t_0,t) - 3) \quad (1)
\]
Using decimal logarithms, this expression is equivalent to

\[ t_S = \frac{0.730 \cdot S(t, t)}{\log N(t) - \log N(t_0)} - 3. \]

Using total cell number and labeling index of endothelium and media and interpolation between 4 and 8 weeks, we obtained an approximate value of 22 hours for the duration of the synthetic phase of the cell division cycle.

Although this is a large value, it falls within the range of previous observations concerning mammalian cells and it is not in contradiction with the observed increase in cell number. Of course it is a crude estimate and it is impossible to take into account the number of cells leaving the population by destruction or migration. Observations of cellular degeneration suggest that this actually does occur. This would mean that, according to the above equation, the duration of the \( S \) phase is in fact shorter than the value we calculated.

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