RADIATION RESEARCH **110,** 118–128 (1987)

Endothelial Alkaline Phosphatase Activity Loss as an Early Stage in the Development of Radiation-Induced Heart Disease in Rats

S. LAUK

Institut für Strahlenbiologie der GSF, Neuherberg, Federal Republic of Germany

LAUK, S. Endothelial Alkaline Phosphatase Activity Loss as an Early Stage in the Development of Radiation-Induced Heart Disease in Rats. *Radiat. Res.* **110,** 118–128 (1987).

Alkaline phosphatase activity of capillary endothelial cells in the heart of Wistar and Sprague–Dawley rats was studied sequentially after single doses of 10, 15, 20, or 25 Gy. Following irradiation capillary density and alkaline phosphatase activity were focally lost before myocardial degeneration or clinical symptoms of heart disease developed. Recovery from both changes took place after doses of 10 or 15 Gy. The decrease in capillary density and enzyme activity showed the same strain difference in latency times and in the extent of the lesions as previously described for pathological and clinical signs of heart disease.

INTRODUCTION

Following local irradiation of the rat heart with doses of 17.5–40 Gy, severe clinical symptoms and all the typical pathological signs of cardiac failure were observed (*1*). The survival time of the animals was dose dependent, ranging from 105 days following 40 Gy to 408 days following 77.5 Gy. Severe focal degeneration of the myocardium was the main histological finding of the disease, and it was suggested that the cause of myocardial degeneration was radiation-induced damage to the capillary network (*1*).

Capillary endothelial cells in the heart contain large amounts of alkaline phosphatase and hence can be labeled in histological sections by a diazo color reaction of the enzyme. Histologically detectable enzyme activity therefore has been used as an assay of endothelial cell function. This report is the first to describe the loss of such activity after local irradiation of the heart. The time sequence of capillary reduction and of myocardial degeneration was determined from the same slides.

Considerable strain differences between Wistar and Sprague–Dawley rats have been reported, both for the latency times and pathology of radiation-induced heart disease (*2*, *3*). To explore the pathogenetic role of capillary damage, the extent and time course of capillary changes have been compared in Wistar and Sprague–Dawley rats.

METHODS

Animals and Irradiation

At an age of 4–5 months male or female rats of either strain were given local irradiation to the whole heart with 300-kV X rays filtered with 0.6 mm Cu and 1 mm Al at a dose rate of 2 Gy/min. Single doses

0033-7587/87 $3.00

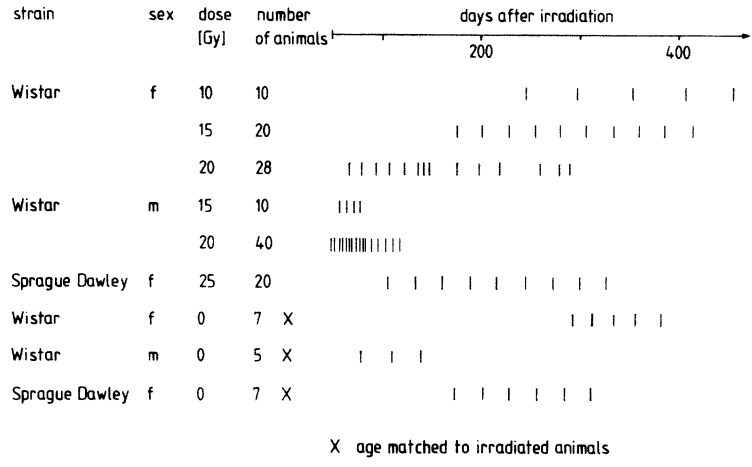

FIG. 1. Summary of the timing of the experiments for the different treatment groups. Every mark corresponds to the examination of two or, in control groups, sometimes of only one animal.

of 10, 15, 20, and 25 Gy were delivered through a lateral field whose position was determined individually for each animal with the help of a lateral chest X-ray picture. Details of the irradiation technique have been described by Lauk *et al.* (*1*). The animals were killed at different times after irradiation. The doses and times of observation were selected based on previous experiments (*1*, *3*). Figure 1 shows the time schedules and the numbers of animals involved. Two animals were examined for each experimental point.

Perfusion, Embedding, and Staining of Tissues

Since the percentage of capillaries in the heart which are actually perfused by blood at any one time may range from 20 to 100% in the individual animal, it is essential for the estimation of volume densities to open all capillaries by perfusion fixation.

At the time of sacrifice the animals were deeply anesthetized with pentobarbital and thoracotomized, and the descending aorta was cannulated retrogradely by an 18-g catheter. The vascular system was flushed by 0.9% saline at a pressure of 140 mmHg. To provide a sufficient outlet for the perfusion fluid, a large mesenteric vein was punctured. As soon as the outflowing fluid was clear, the perfusion was continued using formalin alcohol at a pressure of 130 mmHg. Within minutes the heart was thoroughly fixed. It was taken out, the atria were removed, and the ventricles were cut into six blocks. From each of these blocks at least two randomly oriented slices 0.5 mm thick were taken using two parallel fixed razor blades. The slices were flattened by perforated covers and were kept in 0.1 *M* cacodylate buffer for 4–6 h. They were then transferred through ascending concentrations of glycol methacrylate monomer in ethanol over a period of 3 h. The tissue was then infiltrated with a mixture of methacrylate (40 ml), benzoyl peroxide (0.5 g), and polyethylene glycol 400 (2 ml) for 12 h when polymerization was started by addition of dimethyl sulfoxide (6.6%). The whole rinsing and embedding procedure was carried out at 4°C. Sections 4 μm thick were cut and the alkaline phosphatase was stained by a diazo coupling reaction according to Burstone (*4*). The nuclei were counterstained by nuclear fast red.

Morphometric Analysis

To determine the extent of focal enzyme loss, every section was magnified using a slide projector, and the outlines of the tissue samples were traced on a sheet of paper. The areas of enzyme loss and of myocardial degeneration were identified microscopically and marked on the drawings. The extent of the damaged areas was measured and expressed as a percentage of the total area of all samples from one heart. To investigate capillary changes we have applied morphometric methods to estimate volume density and

length density. The volume density represents the relative volume of tissue occupied by capillaries and thus is related to the volume of blood transported for the supply of the tissue. Length density is an estimate of the total length of capillaries per unit volume of tissue and thus is related to the surface area of capillary walls available for the exchange of substances. Any reduction of the surface area of capillaries, which may impair diffusion through the wall, will be reflected by changes of capillary volume or length density within a given volume of tissue. Volume density was estimated by point counting with a square lattice test system as the following ratio: $V_v = \Sigma P_{cap}/\Sigma P_{myoc}$, where P_{cap} is the number of test points falling on capillaries (consisting of lumen and capillary endothelial cell) and P_{myoc} is the number of test points falling on myocardial tissue.

According to Elias (*5*) length density is given by twice the number of capillary transsections per area examined. A random distribution of fields, however, implies that not every field of vision is completely covered by myocardial tissue. Therefore the examined area of myocardial tissue is smaller than the corresponding field of vision and is estimated by point counting (*6*). This gives the following equation for the calculation of length densities: $L_v = 2 \cdot \Sigma_{cap} \cdot m^2/\Sigma P_{myoc} \cdot d^2$, where cap is the number of capillary transsections per field, P_{myoc} is the number of test points falling on myocardial tissue (reference space), m is the magnification, and d is the distance between test points. This formula applies only to homogeneously distributed, isotropic structures. Since the myocardial capillaries run mainly parallel to muscle fibers, the principle of isotropy does not hold for a single section. However, by using a relatively large number of randomly oriented heart samples per animal it is approximately restored (*7*).

From every heart six randomly oriented samples were taken. The total area of a single section from each of these samples was examined for loss of enzyme activity and myocardial degeneration. The volume and length density of capillaries were estimated from five fields per sample, randomly picked from alkaline phosphatase positive areas. This was possible only in hearts in which the alkaline phosphatase negative area did not exceed 50% of the total heart area.

RESULTS

Loss of Alkaline Phosphatase Activity in Wistar Rats

In the hearts of control animals there is a very dense network of capillaries with muscle fibers and capillaries lying close together. Figure 2 shows that in untreated myocardium all capillary endothelial cells are stained strongly by the alkaline phosphatase reaction. In irradiated hearts a focal loss of the endothelial alkaline phosphatase activity was observed in all dose groups. Figure 3 shows an area of complete enzyme loss neighboring unchanged myocardium. The foci were distributed randomly throughout the myocardium. In every dose group the loss of alkaline phosphatase started in several small foci which later increased in size and number. Areas of myocardial degeneration and necrosis were always located within alkaline phosphatase negative areas and usually covered between $\frac{1}{3}$ and $\frac{1}{2}$ of the area of enzyme loss. On the other hand, even large areas with loss of enzyme activity were frequently observed with obviously normal structure of the myocardium. At 196 and 252 days after 10 Gy the alkaline phosphatase negative areas covered less than 25% of the heart examined and some myocardial degeneration was present. At later times the enzyme activity had returned to normal and the myocardium appeared undamaged. Figure 4 shows the increase in alkaline phosphatase negative areas and in myocardial degeneration with time after 15 Gy in male and female Wistar rats. The first alkaline phosphatase negative areas were found as early as 28 days after irradiation. They seem to increase slowly to a maximum of about 75% at 220 days, with considerable variability between the animals. Later they decreased, but at 360 days, the longest interval investigated, they still amounted to 25 and 13% of the total heart area in the two rats examined. Areas of myocardial degeneration have not yet developed at 28 days and

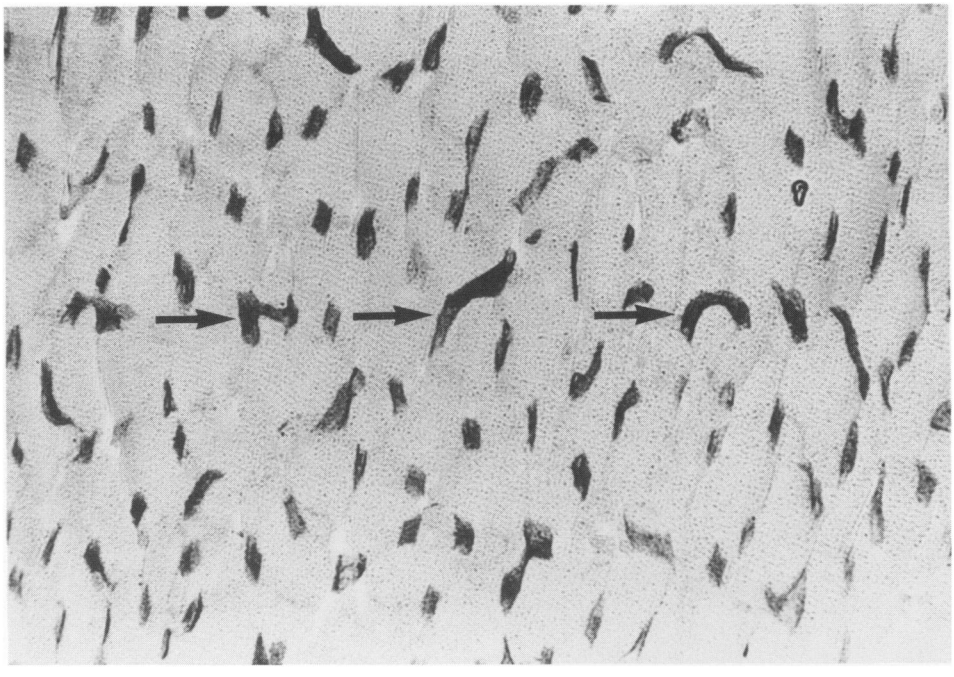

FIG. 2. Section of an unirradiated rat heart. All capillary endothelial cells are stained dark by the alkaline phosphatase reaction. The principal orientation of capillaries is parallel to muscle fibers, which are obliquely cut in this picture. These capillaries are linked by transversal branches (→).

are still rather small at 126 days. Their maximum extent occurs at the same time as maximum enzyme loss covering 25% of the total area, but it appears to recover at longer follow-up times. Figure 5 shows the onset of enzyme loss following 20 Gy, as was examined in both male and female Wistar rats. In both groups a steep increase of alkaline phosphatase negative myocardium, up to 80%, was measured between 25 and 55 days. Degeneration of muscle fibers started later and did not exceed 5% before 70 days. From then on the level of enzyme loss remained high in female rats until death; in males only the first 100 days were examined. During this time there was no difference between male and female animals.

Changes in Capillary Density in Wistar Rats

In untreated male and female Wistar rats the mean volume densities of capillaries were 11.9 and 12.2%, respectively; the length densities were 3262 mm/mm^3 and 3147 mm/mm^3. These values are similar to observations of other authors (*7*). The variation coefficients of volume density between animals were 10 and 8.6% (values for males given first) and of length density were 9.4 and 4.7%. The mean variation coefficients among different samples of one heart were 10.9 and 11.2% for volume density and 17 and 19.4% for length density. This reflects the dependence of length density on the orientation of tissue samples but shows that our sampling procedure de-

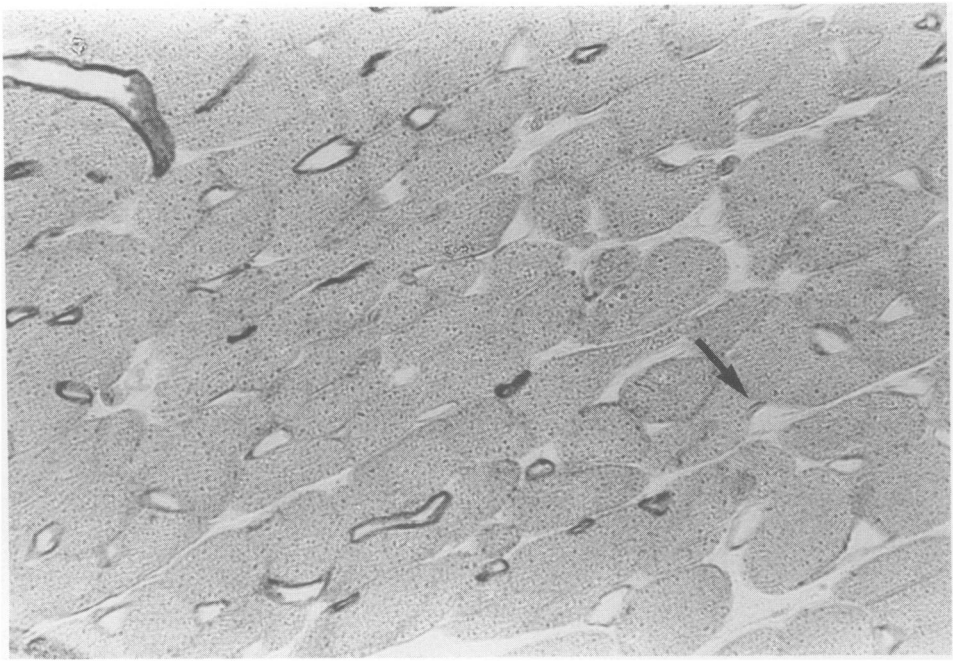

FIG. 3. Section of an irradiated rat heart 224 days after 15 Gy showing the border between an alkaline phosphatase positive area on the left side and a focus of enzyme loss on the right side. The arrow indicates a transverse section of an enzyme negative capillary lined by an endothelial cell with well-stained nucleus.

fines control values rather precisely. Following irradiation the time course of changes in capillary volume and length densities were similar to those of development of alkaline phosphatase negative areas. In all dose groups both volume and length densities

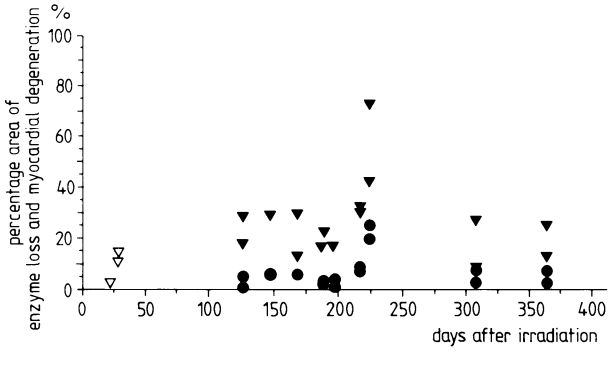

FIG. 4. Development of enzyme loss (▼, ▽) and myocardial degeneration (●, ○) after 15 Gy in female (closed symbols) and male (open symbols) Wistar rats expressed as a percentage of the heart area examined. Each point represents one animal. Experimental points where enzyme loss or myocardial degeneration were not observed are not plotted.

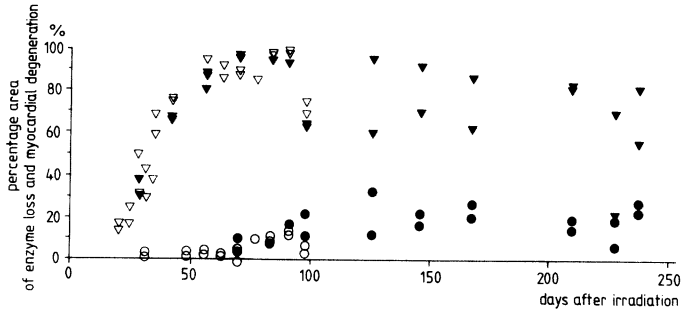

FIG. 5. Development of enzyme loss (▼, ▽) and myocardial degeneration (●, ○) after 20 Gy in female (closed symbols) and male (open symbols) Wistar rats expressed as a percentage of the heart area examined. Each point represents one animal. Experimental points where enzyme loss or myocardial degeneration were not observed are not plotted.

became reduced to less than 70% of control levels. In most cases the changes were parallel, indicating that there was no major modification of capillary shape or diameter.

Following 10 Gy the volume and length densities were already slightly reduced at the initial observation time at 196 days, but they returned to control levels between 300 and 360 days. As represented by Fig. 6, the decrease of volume and length densities measured after 15 Gy started between 50 and 126 days (no measurements were made within this interval). From 250 days on capillary density increased again and reached control levels by 360 days after irradiation.

Figure 7 describes the time course of capillary length density during the first 35 days after 20 Gy. At later times volume and length density could not be determined because then too few alkaline phosphatase positive areas were found to enable selection of a sufficient number of fields in a random way. Both values fell to 50–60% of control levels by 24 days, but these may not be the minimum levels.

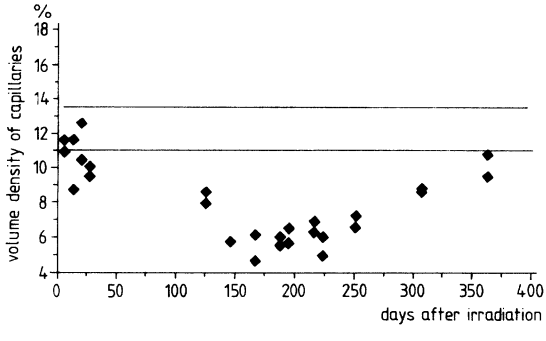

FIG. 6. Variation in time of volume density of capillaries after 15 Gy in Wistar rats. Each point represents the mean value of 30 sections from one animal. The horizontal lines mark the range of the mean values ± standard deviation in control animals.

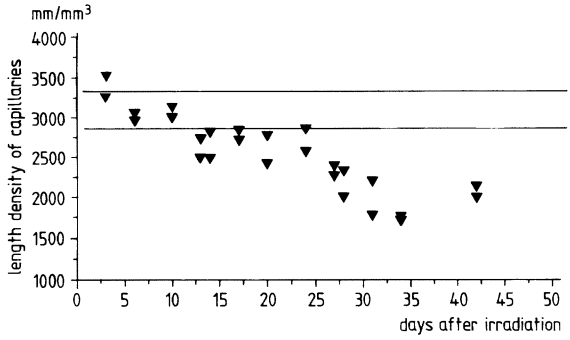

FIG. 7. Variation in time of length density of capillaries after 20 Gy in Wistar rats. Each point represents the mean value of 30 sections from one animal. The horizontal lines mark the range of the mean values ± standard deviation in control animals.

Loss of Enzyme Activity and Changes in Capillary Density in Sprague–Dawley Rats

A comparison of female Wistar and Sprague–Dawley rats showed that the mean volume density of capillaries in untreated Sprague–Dawley rats of 15.5% with a variation coefficient of 6.6% is significantly higher than in Wistar rats. Since there is no strain difference in length density, the cardiac capillaries in Sprague–Dawley rats have a greater diameter than those in Wistar rats.

Figure 8 illustrates that in Sprague–Dawley rats, loss of alkaline phosphatase activity did not occur before 70 days following 25 Gy. Between 120 days and the end of the observation period at 260 days there was no further change in enzyme activity. Only a few small areas of myocardial degeneration were observed throughout the first 8 months after 25 Gy.

In a similar way to the decrease in alkaline phosphatase activity, the volume and length densities started to fall below control levels at 70 days in these animals. The length density remained rather low throughout the rest of the observation period, whereas the volume density increased again from 230 to 260 days (Fig. 9).

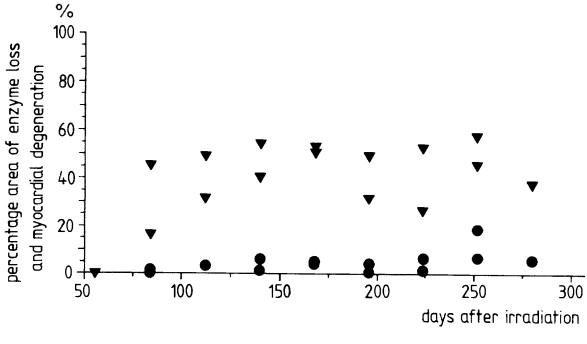

FIG. 8. Development of enzyme loss (▼) and myocardial degeneration (●) after 25 Gy in female Sprague–Dawley rats expressed as a percentage of the heart area examined. Each point represents one animal.

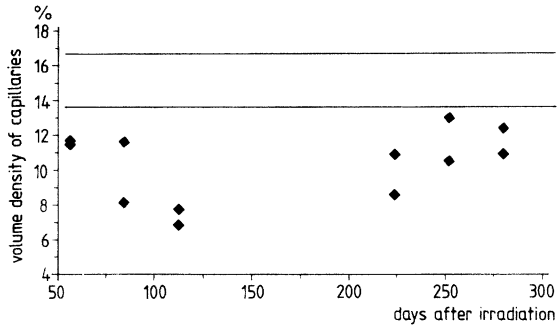

FIG. 9. Variation in time of volume density of capillaries after 25 Gy in female Sprague–Dawley rats. Each point represents the mean value of 30 sections from one animal. The horizontal lines mark the range of the mean value ± standard deviation in control animals.

Significance of the Alkaline Phosphatase Reaction

The alkaline phosphatase reaction carried out in our experiment is unspecific and gives no information on the localization of the reacting enzyme or the role it plays in the function of the myocardium or the capillaries. To gain more information about the actual enzymes affected, we performed the 5′ nucleotidase—and the ADPase—reaction according to the method of Wachstein and Meisel (*8*) in a selection of the material of the present experiment. Both enzymes showed exactly the same distribution of activity in untreated and in irradiated hearts as the alkaline phosphatase reaction. These two enzymes are known to be localized on the surface of endothelial cells. Their function is to degrade ADP to adenosine, which acts as an antagonist to ADP. This way platelet aggregation in capillaries is diminished by these enzymes (*9*). Moreover, adenosine plays a yet undetermined role in the regulation of coronary blood flow (*10*). However, the contribution of the endothelial ectonucleotidases to the myocardial adenosine supply in comparison to other sources of adenosine is not yet known.

DISCUSSION

Irradiation of the heart with doses of 10–20 Gy in Wistar rats and of 25 Gy in Sprague–Dawley rats causes focal loss of the alkaline phosphatase activity of capillary endothelial cells.

For Wistar rats, the maximum extent of enzyme loss is dose dependent and reaches close to 100% after 20 Gy. The latency for onset of focal loss of enzyme activity as well as of decrease of volume and length density of capillaries is similar for 15 and 20 Gy, being around 28 days. The maximum extent of the decrease after 15 Gy is reached around 200 days after irradiation and later both capillary density and enzme activity recover to nearly normal values. After 10 Gy the time course is similar, but the drop in values is significantly less. Defining and counting capillaries by the enzyme activity of endothelial cells is naturally restricted to areas of still positive phosphatase activity. Therefore, following 20 Gy, the decrease in vessel density can be

assumed to be even greater than after 15 Gy, but the sparseness of alkaline phosphatase positive areas precludes meaningful measurements.

Loss of alkaline phosphatase activity appears to be the earliest sign of radiation injury of the capillary endothelial cell. At about the same time a reduction of capillary volume and length density, determined by counting of capillaries lined by alkaline phosphatase positive endothelial cells, occurs.

However, the focal absence of histologically detectable enzyme does not mean that there are no capillaries left in these areas. Figure 3 shows that there are capillaries within alkaline phosphatase negative areas. Moreover, simultaneous labeling of capillaries with India ink perfusion and alkaline phosphatase staining or by PAS and alkaline phosphatase staining of consecutive serial sections in our material demonstrate some patent capillaries with a PAS positive basal membrane in enzyme negative areas. A decrease in the number of capillaries counted could therefore reflect either reduced capillary density in the enzyme positive areas or an additional, scattered loss of enzyme activity in just a few endothelial cells within an alkaline phosphatase positive area. The second possibility is ruled out by the observation that there were never any unstained, morphologically unambiguous capillaries observed in the alkaline phosphatase positive areas. We conclude that loss of alkaline phosphatase activity is focal, while reduction of capillary density is randomly distributed throughout the enzyme positive areas of the myocardium. Therefore point counting of capillaries with positive endothelial cells should give a reliable description of the capillary density in these areas.

The data presented in this paper are supported by earlier attempts to determine the capillary density in PAS stained sections of irradiated hearts (*1*) which gave similar results to the present experiment: the number of capillar transsections was reduced to 85% by 35 days following 20 Gy compared with a reduction of volume density to 83% and of length density to 63% found for the same dose and time in enzyme positive areas of alkaline phosphatase stained sections. Although the rarefication of the capillary network has been claimed repeatedly to be the predominant mechanism for chronic radiation injury in various organs (*11*), very few studies on this topic have been published so far. After irradiation of pig skin with single doses of 1700, 2300, or 2700 R Archambeau *et al.* (*12*) observed an initial increase of the endothelial cell density and the lumen density. This was followed by a steep fall to below control levels from 35 days onward. Since the skin was not perfused but fixed by immersion, only capillaries perfused by blood at the moment of fixation would have been recognized and counted. The initial increase may be explained by an early dilatation of capillaries. Takahashi and Kallman (*13*) irradiated the hind legs of mice with single doses of 2.5 to 40 Gy and then examined the subcutaneous vessels at intervals up to 12 weeks after treatment. Following 20 or 40 Gy there was a continuous decrease of length density from 4 to 12 weeks. Within the same time the vascular volume and the mean diameter of the blood vessels in these animals rose high above control levels suggesting dilatation of surviving vessels. Following doses of 10 Gy and less, no changes of the capillary network were observed. Although these studies were performed on different organs and different species, latency times to decrease of capillary density and effective doses were similar to those of our experiment.

The late recovery of capillary density following 10 and 15 Gy may be caused either by an absolute increase in the number of capillaries or by a decrease of the reference volume of the myocardium. The 50% increase in volume density after 15 Gy (Fig. 5) is not due to a 50% decrease in myocardial volume since these animals had even a slightly increased heart weight in comparison to age-matched controls.

Thus a real structural recovery of the capillary network seems to be going on at very late times after doses of 15 Gy or lower but not after doses of 20 Gy or higher. Extensive myocardial necrosis and subsequent cardiac failure were the late consequences. It is difficult to explain why the enzyme loss should be focal since in general cellular radiation damage occurs at random. Thus it is likely to be a secondary reaction of the capillary endothelial cells to a primary radiation effect in individual endothelial cells. This is similar to observations made in ultrastructural studies in the rabbit heart (*14*) following a single dose of 20 Gy, revealing a maximum of cytoplasmic degeneration of endothelial cells at 20 to 60 days. The changes were observed only in capillary endothelial cells and seemed to be distributed throughout the myocardium.

In Sprague–Dawley rats the latency times for cardiac injuries are much longer and the extent of capillary changes is much smaller than in Wistar rats. The same strain differences were observed for changes in capillary density and endothelial histochemistry as for the clinical course of radiation-induced heart disease and for myocardial degeneration. This supports the concept that radiation acts primarily on the capillary network, while myocardial degeneration and heart failure arise as a consequence of this damage.

To summarize our studies, the sequence of pathological changes in the irradiated heart may be described as follows: Within 4 weeks after irradiation an as yet undetermined expression of injury to discrete endothelial cells leads to focal loss of endothelial cell enzyme activity. These foci subsequently increase by enlargement and fusion as well as by generation of new foci. Enzyme loss in individual endothelial cells or larger areas does not yet imply loss of capillary function nor is it irreversible. However, the diffuse rarefication of capillary density which occurs in enzyme positive areas leads to manifest myocardial necrosis only in those areas of the myocardium which show the loss of enzyme activity.

ACKNOWLEDGMENTS

The author is grateful to Professor K.-R. Trott and Dr. J. Kummermehr for their helpful advice and to Dr. J. F. Knowles for his review of the manuscript. This investigation was supported by Deutsche Forschungsgemeinschaft, project Tr 180/2.

RECEIVED: March 26, 1986; REVISED: November 12, 1986

REFERENCES

1. S. LAUK, Z. KISZEL, J. BUSCHMANN, and K.-R. TROTT, Radiation-induced heart disease in rats. *Int. J. Radiat. Oncol. Biol. Phys.* **11,** 801–808 (1985).
2. T. K. YEUNG and J. W. HOPEWELL, Effects of single doses of radiation on cardiac function in the rat. *Radiother. Oncol.* **3,** 339–345 (1985).
3. S. LAUK, Strain differences in the radiation response of the rats heart. *Radiother. Oncol.* **5,** 333–335 (1986).

4. M. S. BURSTONE, *Enzyme Histochemistry and Its Application in the Study of Neoplasm.* Academic Press, New York/London, 1962.
5. H. ELIAS, A. HENNIG, and D. E. SCHWARTZ, Stereology: applications to biomedical research. *Physiol. Rev.* **51,** 158–200 (1971).
6. E. R. WEIBEL, *Stereological Methods I, Practical Methods for Biological Morphometry,* Academic Press, London, 1980.
7. T. MATTFELDT and G. MALL, Estimation of length and surface of anisotropic capillaries. *J. Microsc.* **135,** 181–190 (1984).
8. M. WACHSTEIN and E. MEISEL, Histochemistry of hepatic phosphatase at a physiologic ph. *Am. J. Clin. Pathol.* **27,** 13–23 (1957).
9. R. BIGGS and C. R. RIZZA, *Human Blood Coagulation, Hemostasis and Thrombosis,* 3rd Ed. Blackwell, Oxford, 1984.
10. S. NEES, V. HERZOG, B. F. BECKER, M. BÖCK, C. DES ROSIERS, and E. GERLACH, The coronary endothelium: a highly active metabolic barrier for adenosine. *Basic Res. Cardiol.* **80,** 515–529 (1985).
11. P. RUBIN and G. W. CASARETT, *Clinical Radiation Pathology,* Vol. 1. Saunders, Philadelphia, 1968.
12. J. O. ARCHAMBEAU, A. INES, and L. F. FAJARDO, Response of swine skin microvasculature to acute single exposures of x rays: Quantification of endothelial changes. *Radiat. Res.* **98,** 37–51 (1984).
13. M. TAKAHASHI and R. F. KALLMAN, Quantitative estimation of histological changes in subcutaneous vasculature of the mouse after x-irradiation. *Int. J. Radiat. Oncol. Biol. Phys.* **2,** 61–68 (1977).
14. L. F. FAJARDO and J. R. STEWART, Pathogenesis of radiation-induced myocardial fibrosis. *Lab. Invest.* **29,** 244–257 (1973).