Inhomogenous capillary flow and its prevention by verapamil and hydralazine in the cardiomyopathic Syrian hamster

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ABSTRACT There is clinical evidence that human dilated cardiomyopathy is related to microcirculatory disorders. We used an experimental preparation of the disease that consisted of a study of the microcirculation of 45 cardiomyopathic Syrian and 18 control hamsters with timed plasma staining. To investigate dynamic vascular disorders a double injection technique was used that permitted demonstration of all permanently and temporarily perfused capillaries in the same animal. The results showed a total capillary density of 3423 ± 470 capillaries/mm^2 in the cardiomyopathic hamster during the premyocytolic phase (30 days of age) and that of 3289 ± 506 capillaries/mm^2 during the myocytolytic phase (44 days). These values were not significantly different from those in the control group (3349 ± 473 capillaries/mm^2 at 30 days and 3383 ± 556 capillaries/mm^2 at 44 days). However, tissue areas with extended coronary transit times were detected only in the cardiomyopathic hamsters. These areas were of the same individual and cumulative size at 30 days (diameter approximately 200 μm, 4% of the tissue) as the myocytolytic zones at 44 days. In cardiomyopathic hamsters verapamil and hydralazine prevented both hypoperfusion and myocytolysis. The results favor the view that microcirculatory disorders generate tissue damage in the cardiomyopathic hamster and that these disorders can be prevented through treatment with the calcium antagonist verapamil or with the vasodilator hydralazine.
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HUMAN dilated cardiomyopathy (DCM) is a heart muscle disease of unknown cause involving, predominantly, impairment of the systolic pump function.[1] The disease carries a poor prognosis; the average yearly mortality rate is approximately 10%.[2–4]

Factor and Sonnenblick[5] suggested that DCM may be caused by microvascular spasm. They pointed to the focal myocardial lesions seen on histologic cross-sections that represented, from their point of view, focal events rather than diffuse abnormalities such as toxic or inborn errors in metabolism.[6]

Although morphologic lesions of the large and small arteries have not thus far been detected, there exists clinical evidence that the disease may be related to impaired blood supply. For instance, patients with DCM exhibit thallium-201 scanning defects during stress testing, indicating myocardial ischemia without coronary heart disease,[7] and also have reduced coronary vasodilator capacity during pharmacologic dilation[8] or ventricular pacing.[9, 10]

In this study, Syrian cardiomyopathic hamsters were used to investigate the microcirculation in DCM. These hamsters were first described by Bajusz et al.[11] and are widely used in experimental preparations because they develop cardiomyopathy and muscular dystrophy in a predictable fashion. Beginning at approximately 30 days of age, the hamsters develop progressive myocytolytic necrosis in heart and skeletal muscle and usually die within 1 year.

It has been suggested that dynamic vascular disorders in these hamsters might affect metabolic supply and cause calcifying myocytolytic lesions.[12, 13] However, structural vascular alterations or endothelial damage before myocardial destruction could not be found.[14] Some authors claim that increased calcium flux across the sarcolemma might lead to cellular hypercontraction, mitochondrial calcification, and cell death.[15, 16]

Verapamil has been shown to prevent myocardial necrosis.[12, 17] If disorders of the microcirculation do

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cause myocytolytic lesions, these disorders should precede the development of myocardial lesions, and since verapamil prevents these lesions, it should also prevent disorders of the microcirculation. Vasodilators without calcium-blocking effects, such as hydralazine, might also prevent these disorders and, if this were shown to prevent myocardial necrosis, it would further support the view that microcirculatory disorders play a part in the pathogenesis of myocytolytic lesions in cardiomyopathic hamsters. Therefore, the aim of this study was to investigate the microcirculation in cardiomyopathic and normal hamsters and to evaluate the effects of verapamil and hydralazine in these animals.

Methods

The experiments were performed on cardiomyopathic Syrian hamsters (CM) of both sexes of the BIO 14.6 strain and on noncardiomyopathic hamsters (NON-CM, controls) of the Stanny Clac strain (both were generously made available by Prof. Dr. Dr. E. Mutschler, Pharmakologisches Institut für Naturwissenschaftler, Frankfurt, West Germany). The animals were fed ad libitum with commercially available food (Altromin). Forty-five CM and 18 NON-CM hamsters were evaluated. Ten CM (body weight 40 to 50 g) and 10 NON-CM (46 to 63 g) hamsters were evaluated at the age of approximately 30 days (29 to 33), and 35 CM (55 to 75 g) and eight NON-CM animals (75 to 94 g) were evaluated at approximately 44 days (42 to 46). The animals were anesthetized with pentobarbital (Nembutal, 0.01 g/100 g ip) and subsequently with ventilation of a 34% O_2 + 66% N_2O gas mixture. The trachea of each animal was cannulated and each was ventilated by means of a Harvard 681 rodent respirator with a constant rate of 45/min and with volume of 1.3 to 1.8 ml. Body temperature was kept constant at 37° C by a heating device.

Arterial blood pressure was measured in the right carotid artery by means of a pressure transducer and a direct writing system. Blood drawn via this arterial catheter was used for blood gas analysis. Drugs were applied via a catheter placed in the right jugular vein.

The thoracic cavity was opened by transsection of the fourth through seventh ribs on the left parasternal side. A small glass cannula was introduced into the left atrium via the left atrial appendage.

For demonstration of those capillaries that had been perfused within a certain period of time, the double dye plasma staining technique of Vetterlein et al.[18] was used. Fluorescein (FITC) and lissamine-rhodamine B200 (RB 200) dyes that had been coupled with bovine serum γ-globulin were used. Details pertaining to the preparation of these solutions are reported by Vetterlein et al.[18]

Perfusion. An FITC-γ-globulin solution (0.35 ml, 7% solution) was slowly infused via the jugular vein catheter. After an interval of 3 min, RB 200 γ-globulin solution (7%) was infused via the left atrial appendage catheter for either 10 sec (0.3 ml) or 1 sec (0.02 ml) by means of an infusion pump and a stopcock. The circulation was then instantly stopped by rapid excision of the heart, which was transferred into precooled isopentane and then liquid nitrogen.

Histomorphometry. The basal surface of the frozen heart was fixed to the mounting stage of a cyromicrotome (Reichert Jung, Friego Cut Mod 2700) at −20° C. Cardiac tissue was removed at the level of the largest diameter in the short axis, perpendicular to the great epicardial arteries of the left lateral wall. At this level, slices of 7 μm thickness were cut and transferred to slides coated with pure alcohol and precooled in liquid nitrogen. The preparations were kept at −20° C until the alcohol impregnated the tissue and sufficient denaturation had taken place. The preparations were then embedded in an artificial medium (Entellan, Merck) at room temperature.

In prior experiments, the mean shrinkage of the slices in pure alcohol was measured to be 3.5% of shrinkage in aqueous solutions.[18] This shrinkage was not considered in the measurements of capillary density reported below.

The 7 μm thick slices were alternatively prepared for epi-illuminative fluorescent microscopy or for transillumination microscopy. For the latter, the frozen slices were lifted off the microtome blade with the slide at room temperature. Thus, the slice thawed instantly at the slide surface and stuck firmly. This material was then stained with hematoxylin-eosin and after dehydration in alcohol and tuluol, embedded in an artificial medium (Entellan).

The unstained slides were observed under a fluorescent microscope with incident light (Zeiss III, RS condensor). Those vessels containing FITC were visualized with a 470/528 nm filter set, and those containing RB 200 with 546/590 nm filters. With use of a 16× oil immersion objective, a 10× eyepiece, and a camera lucida (Zeiss), fluorescent capillaries were indicated on drawing paper. The drawing plate was illuminated by black light (ultraviolet fluorescent tubes) and enclosed in a black box to avoid illumination of the optical system of the microscope. By means of a fluorescent yellow felt-tipped pen whose markings could be seen with the microscope, the outlines of those areas with cross-sectioned FITC- and RB 200-containing capillaries were traced along with the zones within those areas where predominantly FITC-containing capillaries were visible.

The individual capillary positions within those areas were analyzed with a 100× oil immersion objective and a 10× eyepiece and then transferred to drawing paper. With an electronic planimeter (Kontron MOP-AM02), all extensions of predominantly FITC-containing capillary areas and the capillary density were evaluated, taking into consideration the enlargement factor.

All measurements were performed on four individual myocardial slices (two unstained and two stained) from each animal. These slices were obtained from the myocardium of the left lateral wall. In 18 NON-CM and in 20 CM hamsters, additional slices from the interventricular septum were obtained. In each of these slices, one to three randomly chosen areas with cross-sectioned capillaries and muscle fibers were used for the measurement of the capillary density (FITC- and RB 200–containing capillaries separately) and for the measurement of the extent of those areas with reduced RB 200–stained capillary density and with myocytolytic lesions (with transillumination).

Drug dosing in CM hamsters. To evaluate the effect of drugs on myocytolytic lesions and capillary perfusion, three groups of CM hamsters were treated from day 30 to day 44. Verapamil, hydralazine, or normal saline (control group) was applied subcutaneously twice a day in the same injection volume of 0.15 to 0.25 ml, and the evaluator (M. G.) was blinded to the type of treatment being given.

Verapamil group. In a first set of experiments, 20 CM hamsters were treated with verapamil (0.5 mg = 0.2 ml sc twice a day, Knoll A.G.). Six hamsters died within 12 hr, probably due to a faulty injection technique. The injection technique was then modified so that instead of an injection to the dorsal skin, which might injure retroperitoneal organs, the drug was administered via the neck skin. All but one of the remaining animals survived; one animal died on the fifth day. The treatment was continued until day 44, on which six randomly chosen animals were killed and investigated. In a second set of experi-

ments, an additional eight CM hamsters were treated with verapamil. None of the latter animals died. On day 44, four randomly chosen animals were investigated; thus, the results of the verapamil experiments were obtained in 10 animals.

Hydralazine group. Eight CM hamsters were treated from day 30 to day 44 with hydralazine (40 mg/kg body weight = 0.15 to 0.25 ml solution sc, twice a day). Hydralazine (Sigma) was dissolved in 0.9% NaCl solution at a final concentration of 100 mg/10 ml, and then filtrated in a sterile manner. The drug was well tolerated by the animals, and none of them died. The weight gain was equivalent to that in the control group.

Control group. Seven CM hamsters served as a control group. A 0.15 to 0.25 ml sterile 0.9% NaCl solution was injected subcutaneously twice a day. Body weight was 30 to 40 g on day 30 and 55 to 74 g on day 44. No animal died.

Statistics. Histomorphometric measurements from each animal (four to six measurements for the determination of capillary density; four to six measurements for the determination of areas with flow inhomogeneities or myocytolytic lesions) were averaged. For comparison of results either a two-tailed variance analysis or the two-tailed paired Student t test was used. A p value greater than or equal to .05 was considered to indicate the lack of a significant difference.

Results

Total capillary density studied with one dye. To investigate total capillary density throughout the whole left ventricle and in different groups, the values of capillary density obtained after 3 min FITC plasma staining were evaluated separately for the left lateral free wall and for the interventricular septum in the NON-CM and CM groups. As can be seen in figure 1, there were no significant differences in capillary density at two different localizations or between NON-CM and CM hamsters in either age group.

Functional capillary density studied with two dyes. Since in the previous experiments (3 min perfusion) no significant difference in the total capillary density in CM and NON-CM hamsters could be detected, a second dye was introduced to rule out temporarily unperfused capillaries in the CM animals. The second dye test allowed for the counting of capillaries that had been perfused over a short time period in the same animal; with this, we determined the functional capillary density. As can be seen in table 1, there was no significant difference in the capillary density (septum and free wall) observed after 3 min and that after 10 sec of exposure to plasma staining. Therefore, the exposure time for the fluorescent dye RB 200 was further reduced to 1 sec. However, there was again no significant difference from the total capillary density, and this was true in NON-CM and CM hamsters in both age groups (figure 2, *A*).

However, in the CM hamsters there were small localized areas detected where the amount of RB 200–stained capillaries at 1 sec was significantly reduced (table 1; figure 2, *B*). These areas were detectable in all

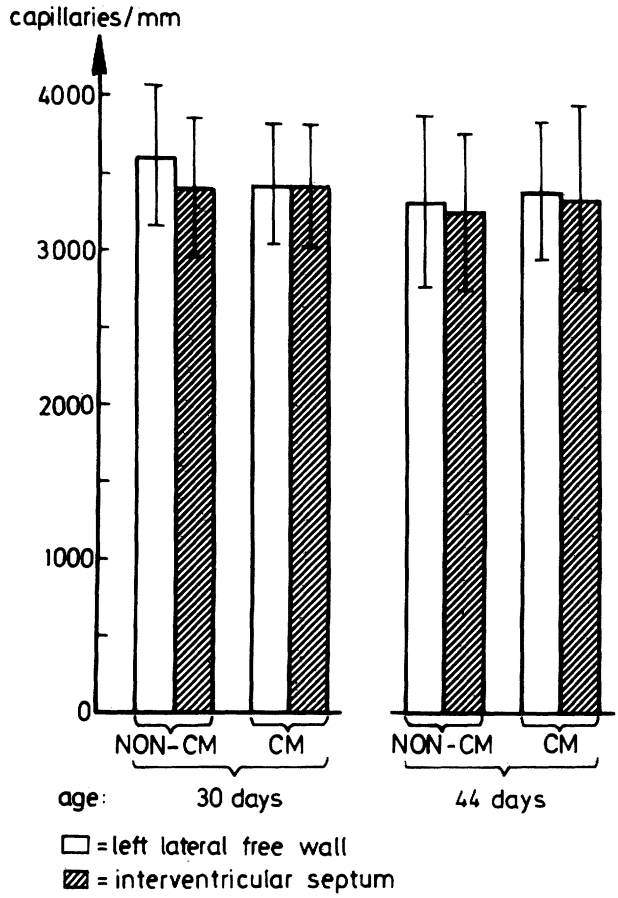

FIGURE 1. Total capillary density measured after 3 min of FITC plasma staining either in the left lateral free wall or in the interventricular septum of CM and NON-CM hamsters at the ages of 30 and 44 days. Note that the capillary density in both groups and at both locations is almost equal. n = 10 NON-CM hamsters at 30 days and n = 8 at 44 days; n = 10 CM hamsters at 30 days and n = 10 at 44 days. The bars represent SDs.

CM hamsters at 30 and 44 days, and were never related to myocytolytic lesions, as could be seen in hematoxylin-eosin–stained cross sections.

Extent of the areas with inhomogeneous capillary staining and myocytolytic lesions. To further characterize the pattern of inhomogeneous capillary staining, the extent of these areas in the fluorescent histologic cross sections was determined. As shown in figure 3, approximately 4% (3.0% to 4.9%) of the tissue in CM animals at 30 days showed staining inhomogeneities (or hypoperfusion at 30 days), while this value declined to 3.56% (2.2% to 5.1%) in CM hamsters at 44 days. Beginning at this age, there were additional myocytolytic lesions seen in hematoxylin-eosin–stained cross sections (figure 3, *right*), that made up about 4% (3.1% to 5.0%) of the area extent.

Fluorescent flow studies of the areas with myocytolytic lesions revealed almost no capillary staining after either 3 min FITC or 1 sec RB 200 staining, indicating

TABLE 1
Capillary density during timed plasma staining in the myocardium of NON-CM and CM hamsters at the age of 30 days (premyocytolytic phase and) 44 days (myocytolytic phase)

	30 days of age						44 days of age					
	FITC, 3 min	RB 200, 10 sec	RB 200, 1 sec Random	p<	RB 200, 1 sec Localized	p<	FITC, 3 min	RB 200, 10 sec	RB 200, 1 sec Random	p<	RB 200, 1 sec Localized	p<
Non-CM												
Mean	3449	3311	3498	NS[A]			3383	3432	3243	NS[A]		
SD	±473	±479	±418				±556	±605	±500			
n	10	4	6				8	4	4			
CM												
Mean	3423	3386	3370	NS[A]	2558	.0001[B]	3289	3119	3339	NS[A]	2372	.0001[B]
SD	±470	±576	±379		±495		±506	±555	±484		±510	
n	10	4	6				10	4	6		6	
p<	NS[A]	NS[A]	NS[A]				NS[A]	NS[A]	NS[A]			

[A]Variance analysis.
[B]Student's t test: FITC, 3 min, vs RB 200, 1 sec, localized.

a reduced total capillary density in certain areas, probably due to capillary degeneration (figure 2, *C*).

Functional capillary density after verapamil, hydralazine, and normal saline treatment. Twenty-five CM hamsters that were treated from day 30 to day 44 with either of the above drugs could be evaluated with respect to total capillary density, functional capillary density, and myocytolytic lesions. As shown in table 2, the capillary density was 3325 ± 596 capillaries/mm^2 (FITC, 3 min) and 3278 ± 568 capillaries/mm^2 (RB 200, 1 sec) in the verapamil-treated group. In the hydralazine-treated group, capillary density was 3380 ± 402 capillaries/mm^2 (FITC, 3 min) and 3388 ± 389 capillaries/mm^2 (RB 200, 1 sec). CM hamsters treated with normal saline solution served as controls: capillary density in this group was 3460 ± 508 capillaries/mm^2 (FITC, 3 min) and 3405 ± 509 capillaries/mm^2 (RB 200, 1 sec) in randomly chosen areas of the histologic cross section. Capillary densities in these sections were not significantly different from those in all other groups. However, in the normal saline group, in contrast to the verapamil- and hydralazine-treated animals, small localized areas with significantly reduced capillary density (2644 ± 547 capillaries/mm^2) were detectable that were equal to those seen in untreated CM animals. These areas of reduced RB 200 staining (1 sec) had an area extent of 3.4% (2.2% to 5.2%) in relation to the whole histologic cross section. As in untreated CM hamsters, additional myocytolytic areas with a cumulative extent of 4.05% were detectable in this group. However, in contrast to the saline-treated and untreated CM animals, in the verapamil- and hydralazine-treated CM hamsters functional flow studies could not demonstrate areas of reduced functional capillary density or myocytolytic areas. Thus, in this study, these pretreated animals did not differ from NON-CM with respect to microcirculation or histology.

Discussion

This investigation aimed to gather further knowledge of the role of microcirculatory disorders in DCM. The experiments were inadequate to deal with the question of whether the results can be extrapolated to human DCM; as mentioned above, however, there is some clinical evidence that microcirculatory disorders do play a role in this disorder.

Although Jasmin and Bajusz,[12] and especially Factor et al.,[13] found evidence that microcirculatory disorders are present in the cardiomyopathic Syrian hamster, neither routine histologic evaluation nor electron microscopy revealed significant structural abnormalities of the vessels before development of or near myocytolytic lesions. Thus, if microcirculatory disorders exist in this setting, one must consider that they are dynamic disorders instead of structural disarrays adjacent to the necrotic zones.

With silicon rubber injections, Factor et al.[13] were able to create a three-dimensional microangiogram of the small arteries and capillaries at the time of death. They found focal narrowing or spasm of microvessels in cardiomyopathic hamsters that could be prevented by verapamil pretreatment. They could not, however, directly demonstrate the effect of vascular narrowing or spasm on plasma flow or the extension of perfusion defects.

With timed plasma staining and the double injection technique, this is possible. The idea is that after 3 or more min of exposure FITC will perfuse all anatomically accessible capillaries (= total capillary density) and no further recruitment can be expected,[18] while exposure to RB 200 for a period of 1 to 10 sec will only result in staining of those capillaries that are being plasma perfused during these times (= functional capillary density). Temporal vascular obstruction or reduced plasma flow velocity will therefore reduce the functional capillary density in comparison with the total capillary density.

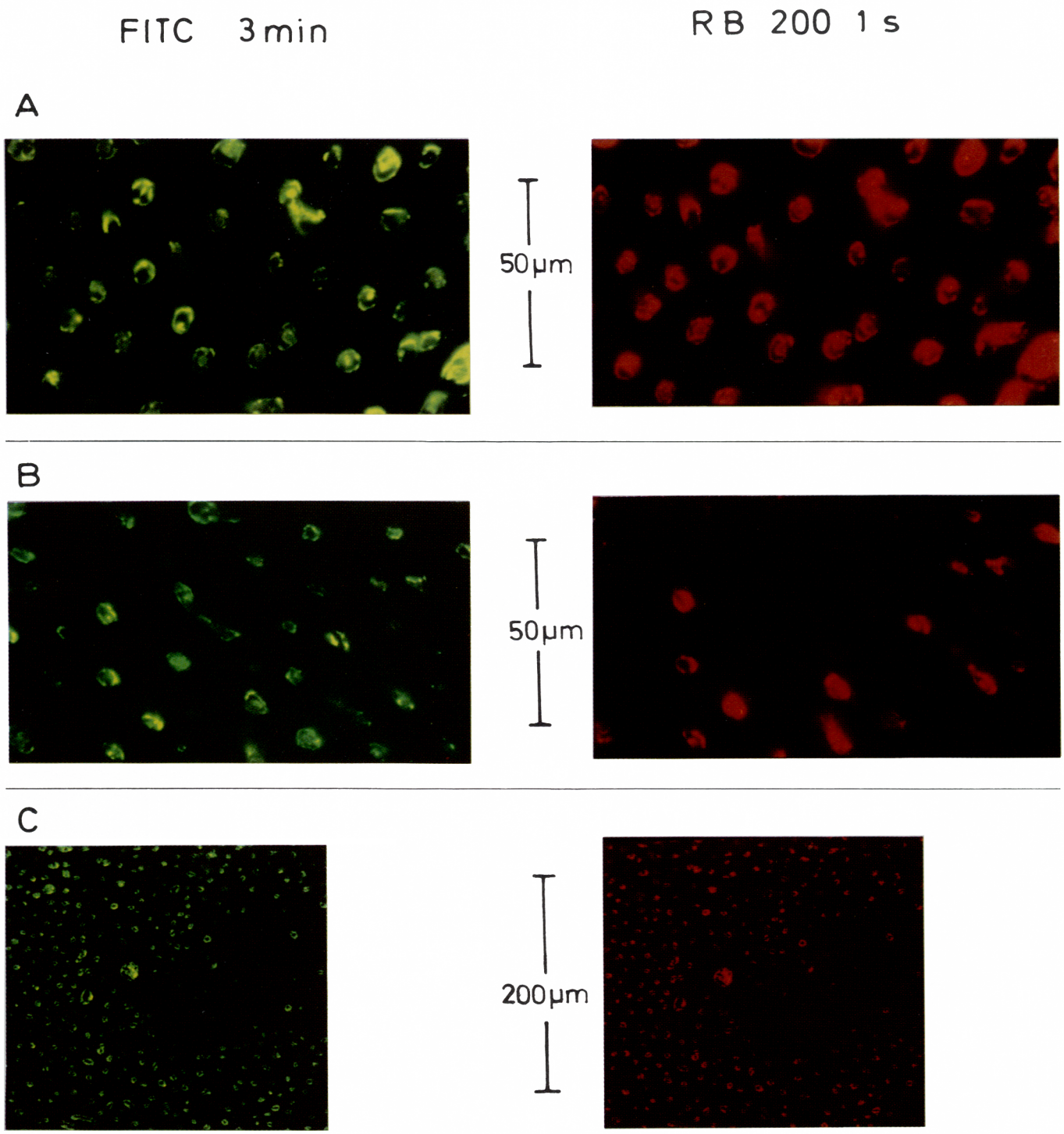

FIGURE 2. Histologic cross sections of CM hamsters plasma stained in vivo. The circulation was exposed to FITC-stained plasma for 3 min (*left*) and to RB 200 for 1 sec (*right*). Photos on the *right* and *left* are of identical areas taken at different fluorescent spectra. *A*, In the randomly chosen areas, every capillary stained over 3 min was also stained at 1 sec. *B*, In localized areas, not all capillaries were plasma perfused by 1 sec. *C*, Localized areas demonstrate no fluorescent capillaries after 3 min or 1 sec plasma staining. Myocytolytic lesions in these areas were demonstrated by the hematoxylin-eosin–stained histologic cross section (see figure 3, *right*).

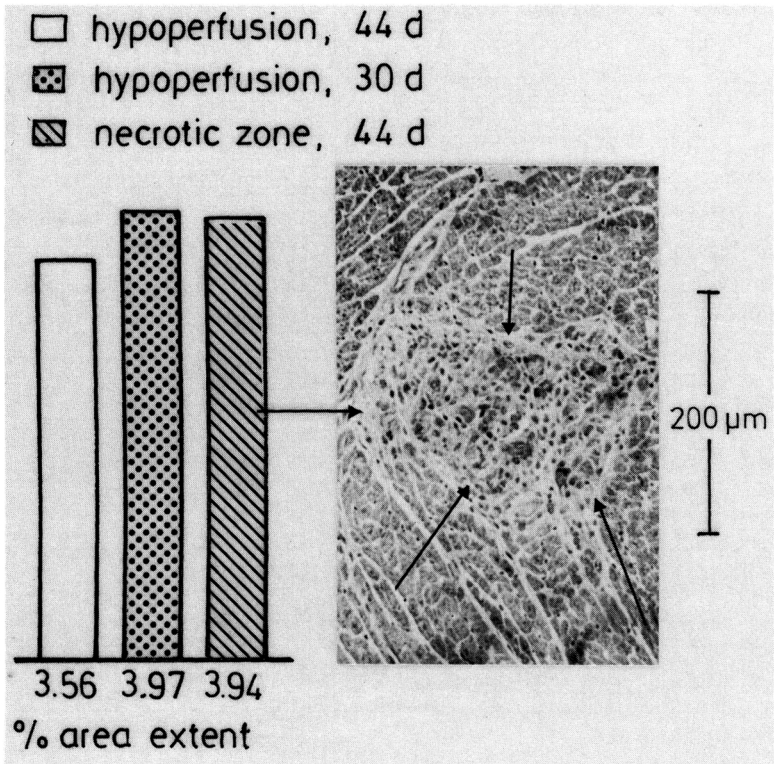

FIGURE 3. Cumulative area extent of zones of hypoperfusion in the CM hamster at 30 and 44 days. Reduced flow is indicated by reduced functional capillary density (1 sec RB 200 staining) and is compared with the area extent of the necrotic zones at 44 days, as demonstrated with hematoxylin-eosin staining (histologic cross section, *right*).

The results show that cardiomyopathy does not affect total capillary density, since density in CM animals resembles that in healthy hamsters and other small mammals.[19] If temporal plasma flow stops due to vasospasm in the CM hamster, the spasm must last less than 10 sec, since application of RB 200 for 10 sec stained all capillaries. When RB 200 was applied for 1 sec, an inhomogeneously stained capillary pattern was detectable only in the CM hamsters, indicating capillary flow inhomogeneities due to an extended coronary transit time of between 1 and 10 sec. These flow inhomogeneities were restricted to discrete zones throughout the myocardium, amounting to a total area extent of 4% at 30 days. Thus, these areas with a diameter of approximately 200 μm did not significantly reduce the overall capillary density of RB 200–stained capillaries.

TABLE 2
Capillary density during timed plasma staining in the myocardium of CM hamsters, 44 days old, pretreated with verapamil, hydralazine, and normal saline

	FITC, 3 min	RB 200, 1 sec			
		Random	p<	Localized	p<
VER					
Mean	3325	3278	NS[A]		
SD	±596	±568			
n	10	10			
HYD					
Mean	3380	3338	NS[A]		
SD	±402	±389			
n	8	8			
SAL					
Mean	3460	3405	NS[A]	2644	.0001[B]
SD	±508	±509		±547	
n	7	7		7	
p<	NS[A]	NS[A]			

VER = verapamil; HYD = hydralazine; SAL = normal saline.
[A]Variance analysis.
[B]Paired Student's t test: FITC, 3 min, vs RB 200, 1 sec, localized.

In former experiments, a significant decrease in the density of stained capillaries was found when the time of dye exposure was reduced to below 5 sec[18]; this was either due to inhomogeneous arrival of the dye front in the arterial network or differences in capillary flow velocity or capillary length, as described by Renkin et al.[20] These experiments were done, however, in rats with a body weight of 90 to 120 g; thus, a longer coronary transit time could be expected. Our experiments were performed in young Syrian hamsters with smaller hearts and a higher heart rate, so that the arrival time of the dye front was earlier and the coronary transit time less. This was clearly demonstrated in the experiments with NON-CM hamsters, in which maximal staining of capillaries was always attainable within 1 sec.

It may be that coronary transit time is affected by a nonspecific epiphenomenon due to hemodynamic impairment in cardiomyopathy; e.g., a reduced transcor-

onary pressure gradient due to elevated end diastolic or intramyocardial pressures. This is, however, quite unlikely, since hemodynamic impairment in this group of hamsters was not detectable in our experiments (blood pressure and pulse rate were normal) and has not been observed in animals of this age by other investigators.[21]

Since treatment with verapamil restores the capillary flow inhomogeneities accompanying cardiomyopathy, it cannot be expected — due to the mode of action of the calcium antagonist[22] — that it will increase the transcoronary pressure gradient (i.e., elevate the arterial pressure or reduce end-diastolic pressure). Also, it is rather unlikely that hydralazine would reduce a possible elevated ultramyocardial pressure, since it would not affect the hyperdynamic circulatory state that might be related to this disease. It is much more likely that verapamil and hydralazine dilate coronary arteries that are obstructed or in spasm. Areas of hypoperfusion were detectable either before the development of myocytolytic lesions or in the myocytolic stage (44 days) of cardiomyopathy, but they did not correspond to myocytolytic lesions (hematoxylin-eosin staining). The area extent of fields of hypoperfu-

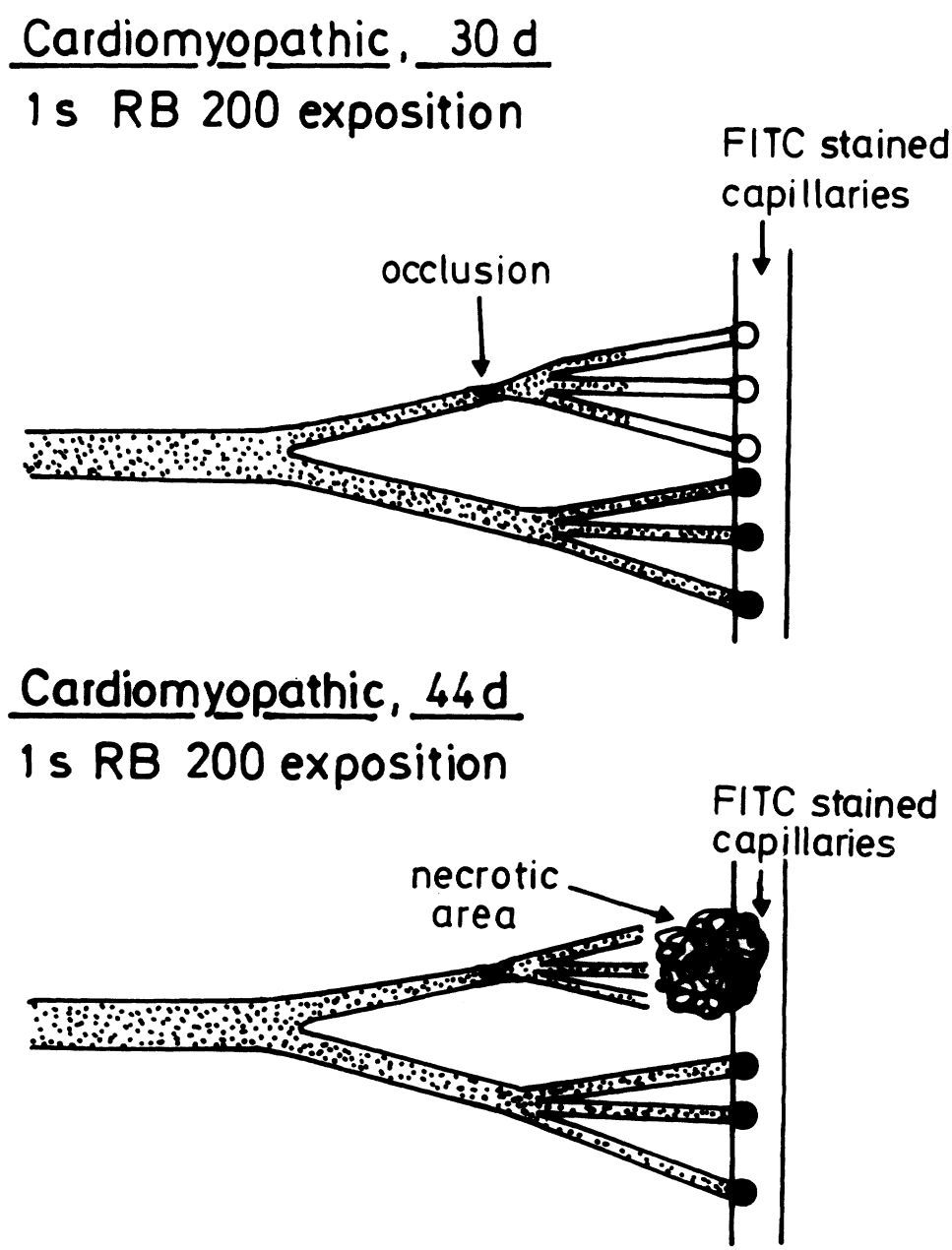

FIGURE 4. Schematic of a possible mechanism of the generation of myocytolytic lesions in the CM Syrian hamster. Occlusion or temporal vasospasm of third-order arterioles delays the dye front; thus, fewer capillaries are stained with RB 200 than with FITC. These areas become necrotic within 14 days.

sion in hamsters 30 days old resembled very closely the extent of the myocytolytic lesions at 44 days. It was recently shown that closure of third-order arterioles results in a lack of capillary perfusion in an area of 200 to 400 μm in the cremasteric muscle.[23] Myocytolytic myocardial micronecrosis without border zones could be induced by 25 μm microsphere embolization,[24] since the heart seems to be organized in end-capillary loops.[25] Thus, ischemia or repeated reperfusion[26] can cause extended myocytolytic lesions equivalent to those seen in cardiomyopathy.

We have demonstrated that there is hypoperfusion in the CM hamster before development of myocytolytic lesions and that the hypoperfused area is in the range of an arteriolar supplying unit. Furthermore, we observed that individual and cumulative sizes of the hypoperfusion zones resemble closely those of the myocytolytic zones and that hypoperfusion and myocytolysis can be abolished precisely and reproducibly by verapamil and hydralazine pretreatment. This favors the view that arteriolar spasm or obstruction causes ischemia with resultant myocytolysis. A schematic diagram of the possible mechanism is shown in figure 4.

According to this view, tissue calcinosis is not the primary event leading to necrosis, but rather the *result* of necrosis. Therefore, treatment with verapamil or hydralazine prevents vascular obstruction (vasospasm) and thus guards against tissue necrosis, as shown elsewhere.[12, 17]

This concept of the cause of myocardial necrosis explains why β-blockers — which do not prevent vasospasm — are ineffective[12] or are less effective[27] in the treatment of cardiomyopathic Syrian hamsters and also why they do not preserve myocardial contractility.[27] On the other hand, in addition to verapamil, α-blocking agents and hydralazine (which are potent vasodilators) reduce the degree of myocardial damage and preserve myocardial contractility in CM.[12, 28] What then generates arteriolar vasoconstriction? This question remains unanswered. However, since there is some evidence that even in human DCM microcirculatory disorders are involved, our results may encourage clinicians to investigate the effect of calcium antagonists, which are free of tachyphylaxis, in patients with this disease.[29] Recent results show that these substances can be applied safely even in high doses in patients with DCM[30]; the initial long-term results in DCM are promising.[31]

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