Table 4. Superoxide dismutase activity in the rural Spanish population, considering the age. Student's t-test was carried out. No significant variations were found.

	Total	18–27	28–37	38–47	48–57	58–65
Sample size	348	81	79	94	67	25
Superoxide dismutase activity mean (Units/ml of blood)	3.81	3.79	3.61	3.89	4.05	3.59
Standard deviation	0.9	0	0	0	0	0

The reason for this increase is unknown. Furthermore, the level in those younger than 18 has not yet been studied; it would be interesting to do this.

Considering the rural population, no significant differences in the superoxide dismutase activity level among different age groups were found (table 4). However, as found by Michelson et al.[16], a small decrease in the superoxide dismutase activity was detected in the rural Spanish population between 58 and 65 years. This little difference could be due to the fact that the number of individuals is smaller in this group. But the hypothesis proposed by Michelson et al.[16] that aging processes occur more rapidly under low superoxide dismutase activity cannot be rejected. This could be due to the modification of repair enzymes, cross-linking and covalent linkage of lipids or nucleic acids to protein caused by an excess of superoxide radical, as well as direct modification of DNA.

Acknowledgments. The authors are greatly indebted to the personnel of the National Institute of Hematology and Hemotherapy in Madrid for their kind cooperation.

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0014-4754/90/080854-03$1.50 + 0.20/0

Increased angiogenesis in diabetes

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Received 21 September 1989; accepted 12 March 1990

Summary. Rats with streptozotocin-induced diabetes mellitus showed a 3.4–4.5 times increased angiogenic response following mast-cell activation in situ as compared with age-matched normal controls. The test tissue used was the mesenteric window, which we have previously exploited as a quantitative angiogenesis assay. In the present study two independent techniques for quantifying the angiogenic response showed essentially the same result. The finding of a pathologically increased angiogenic reaction in the diabetic animals is noteworthy since some of the most harmful complications of diabetes in man relate to proliferative vascular lesions.
Key words. Angiogenesis; diabetes; mast cells; mesentery; quantification; rat.

The most important clinical complications in human diabetes include late developing proliferative lesions in the kidney and retina, as well as advanced atherosclerosis[1]. Examples of such lesions include growth of capillary vessels in proliferative retinopathy[2], one of the most common causes of blindness, the pathogenesis of which is unknown[3,4]; proliferation of smooth muscle cells; and the formation of new vessels in atherosclerotic plaques in advanced atherosclerosis[5].

As we have demonstrated earlier, rats suffering from 4 weeks of experimental diabetes show an augmented mast-cell-mediated mitogenic reaction in various cell types in the test tissue used in the present study, the mesenteric window, in vivo[6,7], as well as in organ culture[8]. This augmented cell proliferation is unaffected by insulin[6,9] and is not due to hyperglycemia per se[10]. Increased mitogenesis appears to be causally related to some cellular and/or extracellular factor that slowly manifests its effect during the course of the disease[8]. Mast cells are not only able to stimulate surrounding cells to synthesize DNA and divide, by a paracrine mode of action in normal and diabetic tissues, but also, when

activated in situ, they can induce angiogenesis, i.e. the formation of new blood vessels, in normal rats and mice[11–13].

In the present investigation the angiogenic response in mesenteric windows following local mast-cell secretion[11–15] in rats with streptozotocin-diabetes of 4 weeks' duration was studied by two quantitative methods that rely on independent techniques: the number of vessel profiles per unit length in perpendicular sections of this extremely thin membranous tissue, and the area of vascularization in spreads of intact mesenteric windows.

Materials and methods

Animals. Male Sprague-Dawley rats (Alab AB, Sollentuna, Sweden), weighing (mean ± SE) 186 ± 1.6 g at the start of the experiment, were used. They were conditioned, stored and fed water and standard pellets ad libitum, as described elsewhere[11].

Mesenteric windows as test tissue. In the rat the true mesentery contains some 40–45 translucent membranous windows[16,17] which are about 5–10 µm thick[18]. Because the tissue is so extremely thin the entire cellular composition can be analyzed by light microscopy in unsectioned spreads. Mesothelial cells and fibroblasts each constitute approximately 46–50 %, mast cells 2–3 % and macrophages about 1–2 % of all the tissue-bound cells[18]. The windows contain few naive blood vessels[11,12]. They were used as test tissue since they allow the quantification of the microvasculature present (see below), and since extensive knowledge of this tissue is available from previous studies in this laboratory on mast-cell-mediated mitogenesis in normal and diabetic rats.

Quantification of blood glucose. Blood samples from the tip of the tail were analyzed using the glucose-oxidase-peroxidase technique[19].

Diabetogenic treatment. Streptozotocin (Sigma Chemical Co., St. Louis, MO, USA) was injected into a tail vein in a single dose of 65 mg/kg[20]. Animals that showed a blood glucose concentration that was more than 3 times the normal level were considered to be diabetic (table 1). These animals showed a reduced body weight gain compared with the age-matched controls (table 2). There was one diabetic rat per cage and as a rule it shared the cage with one control. No insulin was given. No animal died spontaneously.

Mast-cell secretion as an angiogenic effector. Compound 48/80 (Sigma), a polymer obtained by heating 4-methoxy-N-methylphenethylamine and an equimolar quantity of formaldehyde in an acid solution[21], has long been used as a pharmacological tool to induce selective mast-cell activation in many species. It is often referred to as the archetypal histamine liberator[22]. As discussed elsewhere, 48/80 is angiogenically and mitogenically inert in the mesenteric window when tested in guinea pigs, the mast cells of which are unresponsive to the drug[11]. In the present study the diabetic and control rats were subjected to i.p. treatment with 48/80 four weeks after the diabetogenic streptozotocin-treatment. The drug was dissolved in saline (0.9 %, w/v, NaCl) and injected i.p., 2 ml/100 g b.wt daily at 8.00 a.m. and 3.00 p.m. for 2½ days to give a total of 5 injections, starting at 2 µg/g b.wt and increasing by 1 µg/g b.wt each day; untreated normal and diabetic animals were kept as controls. This treatment induces a conspicuous angiogenesis in normal animals as measured in mesenteric windows[11–14]. The experiment ended 14 days after the start of the i.p. treatment. Since the angiogenic response when examined on day 14 in normal animals increases markedly with the number of days of the i.p. 48/80-treatment given – for example, it is considerably greater after 4.5 than after 3 days of treatment[13] – the response in the controls observed in the present study after 2.5 days of 48/80-treatment should be far from the maximum.

Table 1. Blood glucose concentration in the diabetic rats and age-matched controls one week after the streptozotocin treatment. Mean ± SEM.

	Blood glucose mmol/l
Control (20)	4.94 ± 0.07
Diabetes (28)	18.48 ± 0.07

Figures in parentheses indicate the number of animals.

Table 2. Body weight (g) of the normal controls and the diabetic animals at the time of the start of the i.p. treatment with compound 48/80 (day 1), after the completion of the treatment (day 3) and on day 14 when the experiment was concluded. Mean ± SEM.

	Day 1	Day 3	Day 14
Control (20)	348.8 ± 6.7	345.0 ± 9.6	405.8 ± 9.4
Diabetes (28)	191.0 ± 6.2	187.9 ± 6.0	206.0 ± 6.9

Figures in parentheses indicate the number of animals.

Visualization of mesenteric-window vessels by enzyme-histochemistry. We modified an ATPase incubation technique previously used to visualize vessels by demonstration of Mg^{2+}-activated ATPase in canine retina[23]. Intact mesenteric windows were spread on objective slides pretreated with poly-L-lysine. The specimens were then fixed in buffered neutral 4 % (w/v) formalin solution at 4 °C for 1 h, then rinsed 4 times for 5 min each in tap water at 4 °C before they were incubated in a freshly made medium containing 0.2 M tris-maleate buffer, pH 7.2, that had been filtered through paper and warmed to 37 °C. Just prior to placing the spreads in the medium 1 mg ATP (adenosine 5'-triphosphate, grade I crystalline sodium salt; Sigma) per ml was added to the medium. The spreads were incubated for 30 min in a gently rocking water bath at 37 °C. The specimens were then washed 5 times in tap water at room temperature and treated in a 1 : 100 ammonium sulfide solution (light solution; Merck, Darmstadt, FRG) for 1 min in order to convert the lead phosphate reaction product to lead sulfide. The specimens were then washed several times in tap water at

room temperature, dried and analyzed morphometrically without mounting (see below).

Quantification of angiogenesis in mesenteric windows. The *number of vessel profiles per unit length* of the tissue was counted microscopically at × 1000 in 3 µm-thick perpendicular sections of methacrylate-plastic embedded window, as described elsewhere[14,16]. Three or four (mean ± SEM = 3.84 ± 0.10) windows per animal were used. In each of the somewhat triangular-shaped windows the tissue was sampled close to the periphery at either of the two 'corners' adjacent to the small gut.

The *relative vascularized area* in 2 or 3 (mean ± SEM = 2.19 ± 0.07) intact windows from each animal after the ATPase incubation procedure was analyzed morphometrically in spreads at × 40 as described elsewhere[14].

There was no significant difference in the size of the normal and the diabetic windows examined.

Statistics. The difference between means was assessed using Student's t-test; $p \leq 0.05$ was considered significant.

Results

Number of vessel profiles per unit length of mesenteric window. As shown in table 3, the relative increase in the 48/80-treated diabetics exceeded the relative increase in the 48/80-treated controls by some 3.4 times; the difference was highly significant in statistical terms. The mean number of vessels in the untreated diabetic animals was about half of that seen in the untreated controls ($p \leq 0.30$; table 3). The 48/80-treatment increased the number of vessels in the controls by about 3 times and by about 11 times in diabetics; following 48/80-treatment the number of vessels was 72% higher ($p \leq 0.10$) in the diabetic animals than in the controls.

Table 3. Number of vessel profiles/mm of mesenteric window in untreated and 48/80-treated diabetic animals and age-matched normal controls. Sixteen 48/80-treated and four untreated controls as well as twenty-three 48/80-treated and five untreated diabetic rats were used. There was no statistically significant difference between the untreated controls and the diabetic animals. Treatment with the mast-cell secretagogue 48/80 i.p. induced a conspicuous, statistically significant increase in vessel number in both the controls (3.1-fold increase; $p \leq 0.001$) and the diabetic animals (10.6-fold increase; $p \leq 0.001$). The relative increase in the number of vessel profiles following 48/80-treatment was thus approximately 3.4 times greater in the diabetics than in the controls. The number of vessels was normalized by setting the mean of both the untreated controls and the untreated diabetics at 100, and then expressing every individual value from the 48/80-treated as well as the untreated animals in each of the control and diabetic groups as a percentage of this mean. When comparing the normalized figure of the mean (± SEM) of the 48/80-treated controls (306.9 ± 49.4) and the 48/80-treated diabetics (1058.8 ± 193.5) $p \leq 0.005$. Mean ± SEM.

	No. of vessels/mm			
	Control		Diabetes	
Treatment		% increase		% increase
None	0.336 ± 0.147 (15)		0.170 ± 0.079 (20)	
48/80	1.031 ± 0.166 (63)	307	1.808 ± 0.319 (83)	1059

Figures in parentheses indicate the number of window specimens.

Table 4. Percentage of vascularized area in mesenteric window of untreated and 48/80-treated diabetic animals and in untreated and 48/80-treated age-matched normal controls. The untreated diabetic animals showed a significant reduction in vascularized area compared with the untreated controls ($p \leq 0.02$). The 48/80-treatment i.p. caused a strong angiogenesis in both types of animal ($p \leq 0.001$), the increase was in fact 5.7 and 25.9 times in the normal and the diabetic animals, respectively. The relative angiogenic response was thus about 4.5 times greater in the diabetic animals. The values were normalized in the same way as in table 3. A comparison of the normalized figure of the mean (± SEM) for the 48/80-treated controls (575.0 ± 71.6) with that for the 48/80-treated diabetics (2590.1 ± 419.4) showed $p \leq 0.001$. Mean ± SEM.

	Vascularized area (%)			
	Control		Diabetes	
Treatment		% increase		% increase
None	4.57 ± 1.43 (11)		0.92 ± 0.37 (13)	
48/80	26.26 ± 3.27 (24)	575	23.83 ± 3.86 (20)	2590

Figures in parentheses indicate the number of window specimens examined.

Vascularized mesenteric-window area. As shown in table 4, the relative angiogenic response was approximately 4.5 times greater in the diabetic animals than in the controls; the difference was highly significant in statistical terms. The untreated diabetic animals demonstrated a marked, statistically significant reduction in the vascularized area in comparison with the untreated controls (table 4). The diabetic animals which had been exposed to 48/80 showed an increase of almost 26 times, whereas the increase in the control animals after exposure to 48/80 was close to 6 times.

Discussion

The main finding in the present study was a markedly increased mast-cell-mediated angiogenesis in rats with streptozotocin-induced diabetes, a recognized model for type I diabetes[1]. It is most unlikely that extrapancreatic side effects of streptozotocin could account for the increased angiogenesis developing during a 14-day period following selective mast-cell secretion starting 4 weeks after a single diabetogenic streptozotocin injection, as observed here. The augmented angiogenic response in the diabetic animals is notable since microvascular proliferative lesions are frequent in human diabetes[1-5].

On activation the mast cells release a series of potent mediators including factors of potential importance in angiogenesis such as heparin, histamine and a variety of proteases, affecting nearby cells and the extracellular matrix in a complex way, as discussed elsewhere[18,24]. Moreover, angiogenesis, irrespective of the initiating factor involved and the tissue studied, is a complicated reaction engaging several types of cell and matrix components, and developing in a series of sequential steps[25-27]. With the angiogenesis effector used in the present paper[11], i.e. the activation of autogenous mast cells in situ by a highly selective secretagogue, we essentially study features related to mast-cell function in normal and diabetic tissue. Since there is practically no physiological angiogenesis in the test tissue[12] the response

studied relates to true neovascularization. The molecular mechanisms of the mast-cell-mediated angiogenic reaction are as yet unknown. It is, however, only atoxically suppressed over a narrow concentration range by protamine sulphate [28] which is, among other things, a specific antagonist to heparin. Mast-cell-mediated angiogenesis, which is age-dependent [12] and dose-dependent [13], can, moreover, be influenced by hormonal stimuli [15]. In passing, it may be mentioned that 48/80-induced mast-cell activation in the embryonic chick chorioallantoic membrane can enhance ongoing organogenic angiogenesis [29].

In rats with streptozotocin-induced diabetes, the mesenteric windows are changed hyperplastically in terms of the increase in area, number and content of DNA, mast-cell histamine and protein [16, 17]. (A remarkably similar mesenteric-window hyperplasia occurs also in normal lactating rats [30], which like the diabetics show hyperphagia.) However, the content per mast cell of preformed mediators such as histamine, 5-hydroxytryptamine and heparin are unaffected by diabetes, as are mast-cell histamine concentration and 48/80-induced histamine release (a marker of mast-cell secretion) in the test tissue [6]. This argues against mast-cell hyperactivity as a cause of increased angiogenesis in diabetes.

The reduced vascularity in the windows from the untreated diabetics in comparison with the untreated controls may relate to the hyperplastic state of the tissue or to the loss of microvasculature, as has been observed in other tissues in experimental and human diabetes [31–33]. The lower capillary density in skeletal muscle of diabetics is, for instance, believed to reflect capillary degeneration and inhibition of capillary proliferation in the diabetic state, as discussed recently by Lash et al. [33].

As mentioned earlier, rats that suffer from streptozotocin diabetes of 4 weeks' duration show an augmented mitogenesis in connective tissue and epithelial cells following local mast-cell secretion in various tissues [6, 7, 34, 35]. Evidence indicates that some change in the test tissue itself, slowly acquired during the diabetic disease, rather than some systemic factor or the hyperplastic state of the tissue, is causally linked to the augmented mitogenesis [8–10, 34, 36]. Whether the duration of diabetic disease influences mast-cell-mediated angiogenesis has yet to be elucidated.

The design and verification of specific, reliable, repeatable and precise methodology to measure angiogenesis is considered an imperative of high priority in the field of angiogenesis research [37]. The versatility of the quantitative rodent mesenteric-window angiogenesis assay that was introduced recently [11–15, 28] remains to be defined fully. Nevertheless, it is fair to say that both methods used for quantifying the angiogenic response in the present study have unrivaled features.

In man, vascular lesions play an important pathogenic role in diabetes complications such as myocardial infarction – the most common cause of death in diabetics – diabetic nephropathy, and proliferative retinopathy [1, 5, 38–41]. Our finding of an increased angiogenic response in diabetic animals is therefore not only interesting but also promising in the search for good models for the investigation of disturbances in angiogenesis in diabetes. Obviously, this experimental system allows the effect of different anti-diabetes treatment protocols to be tested. Further investigation will, moreover, reveal whether the diabetic mesenteric window, as used here, is a particularly sensitive and potent angiogenesis assay, not only for secreting autogenous mast cells, but for discrete angiogenic factors as well.

Acknowledgments. We wish to thank Mrs Gunvor Jefferth and Miss Ann Nehlmark for their excellent technical assistance and Mrs Erna Johansson and Mrs Ingrid Johnsson for running the animal stable so excellently. This work was supported by grants from the Swedish Medical Research Council, project no. 5942, the King Gustav V Jubilee Clinic Cancer Research Foundation at Sahlgren's Hospital and the Assar Gabrielsson Foundation for Clinical Cancer Research. The experiments have been approved by the Experimental Animal Ethical Committee in Gothenburg.

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0014-4754/90/080856-05$1.50 + 0.20/0

Evidence for a modulation of the stress response by the pineal gland

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Received 22 September 1989; accepted 5 December 1989

Summary. Wistar rats show a circadian variation in their response to stress. Pinealectomy exacerbates stress-induced gastric ulceration in rats. This effect is counteracted by melatonin administration.
Key words. Pineal gland; stress; melatonin.

The mammalian pineal gland is innervated by autonomic postganglionic sympathetic fibres, originating in the superior cervical ganglia [3]. It synthesizes and releases a hormone, melatonin, which has been implicated in the functional activities of many organs, including the adrenal glands [4]. A variety of studies have shown that exposure of rats to stressful stimuli alters melatonin production [5–7], and there is evidence for a stimulation of pineal N-acetyl transferase (NAT) activity in certain stressful situations [5]. NAT is an important enzyme necessary for melatonin synthesis. Miline [8] has reported results indicative of a functional antagonism between the pineal gland and the pituitary, and suggests that the pineal gland participates in the general adaptation syndrome in stress. Studies have shown that the pineal gland modulates adrenal steroidogenesis in normal as well as stress conditions [9]. Mehdi and Sandor [10] suggest that melatonin may be involved in a modulation of corticosteroidogenesis. Dickson and Hasty [11] proposed that the pineal gland secretes a substance that acts at the level of the hypothalamo-pituitary axis to reduce the production and release of adrenocorticotropic hormone (ACTH), with a resultant inhibitory influence on the pineal gland. Thymus involution can be used as a marker of exposure to stress [12]. Maestroni et al. [13] recently reported that melatonin inhibits the effects of stress on thymus weight, acting via an opiatergic mechanism. Gastric stress ulcer formation is an obvious peripheral consequence of exposure to prolonged stress [12]. An early study by Senay and Levine [14] showed that prone restraint on a board, coupled with exposure to cold, acts synergistically to rapidly produce gastric ulceration in starved rats. Using a similar method, a preliminary study done in our laboratory showed that melatonin significantly inhibits the formation of gastric lesions in stressed rats [15]. This study was thus undertaken to investigate the possibility of a modulation of the stress response by the pineal gland.

Materials and methods

Animals. Inbred female rats of the Wistar strain, housed five per cage, were used in all experiments. Animals were maintained under an automatically regulated lighting cycle of LD 12:12 with lights on at 06.00 h. They were allowed free access to a standard diet and tap water prior to experimentation.

Drugs. Melatonin (Sigma) was freshly prepared in a vehicle containing 2% benzyl alcohol, 10% polysorbate 80, and 0.025% w/v citric acid, and made up to volume with distilled water.

Stress. Rats were deprived of food for 24 h prior to restraint, but allowed access to water ad libitum. Coprophagy was prevented by using cages with steel grid bases, elevated from a receptacle in which faeces accumulated. After the starvation period, animals were lightly anaesthetized with ether, injected with treatment drug or control vehicle (see experiments) and immediately restrained in the supine position on a wooden board, using adhesive tape. By this time the rats had recovered from ether anaesthesia and were immediately transported to a cold room (4–7 °C) where they remained for the duration of the stress procedure. During the daytime studies, the light intensity in the cold room was 600 lux, sufficient to inhibit endogenous pineal melatonin synthesis [15]. After two hours, the rats were removed from the cold room