Cyclosporine is angiostatic*

K. Norrby

Department of Pathology, University of Göteborg, S-413 45 Göteborg (Sweden)
Received 1 April 1992; accepted 19 August 1992

Abstract. The systemic effect of the immunosuppressive drug cyclosporine (CS) on formation of new blood vessels was studied quantitatively in rats using the mesenteric-window assay. Angiogenesis was induced by i.p. injection of saline. CS at a s.c. dose of 4 mg/kg/day, which is in the range used clinically, suppressed angiogenesis (inhibiting branching or tortuosity more than spatial expansion), and appeared to be non-toxic. This is the first report on an apparently selective angiostatic effect of CS. The finding is likely to have implications for the clinical use of CS, not only in certain types of organ transplantation but possibly also in psoriasis and other angiogenesis-dependent diseases.
Key words. Angiogenesis; cyclosporine; rat; systemic effect; quantitation; mast cells; blood glucose.

The cyclic undecapeptide cyclosporine (CS) molecule is involved in a fairly wide spectrum of biological activities including anti-parasitic effects, but it is mainly known for its exceptional immunosuppressive properties and is widely used in organ transplantation[1]. The immunosuppressive capability of CS is thought to be based on its ability to inhibit the production of lymphokines (e.g. interleukin 1 and 2) and to inhibit the responsiveness of T lymphocytes to these lymphokines. Through mechanisms which are as yet unclear, CS also induces a clinical improvement in skin lesions and arthritis in patients with psoriasis[2-5]. Moreover, CS appears to exert an anti-tumour effect with respect to the spontaneous occurrence of tumours in rats, and certain clinical and experimental epithelial cancers[1,6-8], although its suppression of cell-mediated immunity should favour tumour growth rather than tumour regression. Since angiogenesis, the development of new blood vessels, is a characteristic of psoriatic skin and arthritic lesions as well as prerequisite for solid tumour growth, we studied the possible systemic anti-angiogenic effect of CS.

The mesenteric-window assay was used to quantify the systemic effect of CS on de-novo microvascular formation in adult rats. Important aspects of the method are that a) the test tissue, which is normally sparsely vascularized, virtually lacks physiological angiogenesis[9], like most tissues that show clinically relevant angiogenesis, b) the assay is truly quantitative, which is unique, and, c) the assay is very well suited to the study of the systemic effects of angiogenic and anti-angiogenic factors in a mammalian system[10-12]. We found that CS at an apparently atoxic dose of 4 mg/kg/day, which is in the dose range used therapeutically in patients, significantly suppressed angiogenesis.

Material and methods

Animals. Adult male Sprague-Dawley rats (Alab AB, Sollentuna, Sweden) fed standard pellets and water ad libitum were used. Two animals shared each cage. During $\geq$ 5 days prior to experimentation the animals were acclimatized to the standardized conditions in which they were kept during the entire experimental period of 14 or 16 days[13]. The animals were weighed at least every other day during the experimental period which included the i.p. injection of either saline or the highly-selective mast-cell secretagogue compound 48/80 (Sigma; see below); 48/80 was included since it interferes transiently with the normal body-weight gain[13] and thereby makes the test of the effect of CS on body-weight gain more stringent (see below). The mean body weights at day 0 were 192 and 224 g in the two experiments done. The controls gained 48 and 49% in weight during the two experiments, compared with their weight at day 0.

Cyclosporine. Cyclosporin A (CS), infusion concentrate (Sandimmun®; Sandoz), a fungal, highly lipid-soluble 11-amino acid polypeptide with a molecular weight of 1202, was diluted with isotonic saline (0.9%, w/v, NaCl; B. Braun, Melsungen AG, Germany) and injected s.c. once daily (at 07.00–07.30 h) from day 1 through days 13 or 15, i.e. up to the day before sacrifice. Doses of 4, 8 and 16 mg/kg/day (in about 0.7 ml) were injected s.c. into the back. The s.c. route was chosen since it provides reproducible and steady CS plasma levels with little variation over a 24-h period in rats[14]. CS is well distributed in almost all tissues and metabolized in the liver to some 15 metabolites, some of which may have an immunosuppressive effect.

Angiogenesis assay. The mesenteric-window assay was used to quantify the vasculature by two technically-independent modes, both of which show a remarkably high degree of sensitivity and reproducibility[10]. From every animal 4 windows were examined using each technique; there was no significant difference in the mean size of the windows used in the different experimental groups (data not shown). a) The *number of vessel profiles per unit tissue length* in 3-µm-thick, toluidine-blue-stained sections, examined microscopically at × 400[13,15], is a measure not only of the degree of branching or tortuosity but also of the spatial extension of the microvascular tree. The number is given per 25.6 mm. b) The *relative vascularized area* was measured morphometrically in spreads of whole, unsectioned windows[16], after the vasculature had been

visualized by enzyme histochemistry[17]. This variable, which represents the vascularized area in percent of the whole window area, relates primarily to the spatial extension of the essentially two-dimensional vasculature.

Induction of angiogenesis. To induce mesenteric-window angiogenesis, isotonic saline (0.9% NaCl; Braun) was injected i.p. twice daily for 4 1/2 days, starting on day 0[13]. This treatment, which does not significantly activate the mast cells in the test tissue[18], causes significant angiogenesis[16]. The molecular mechanism of this type of angiogenesis is not known, but saline possibly exerts its angiogenic effect because of its low content of endotoxins which, however, does not exceed the quantity accepted by the European Pharmacopeia for human use, as tested in rabbits.

Quantification of blood glucose. After 9 days of CS treatment, blood was withdrawn from the tip of the tail and analysed by a glucose oxidase peroxide method[19].

Quantification of histamine release as a marker of mast-cell secretion. In the test tissue the mast cell is the major, if not the only, repository of histamine. The histamine base per protein (ng/μg) was measured 2 h after an i.p. injection of compound 48/80 using a fluorometric method[20].

Statistics. The non-parametric Mann-Whitney U rank sum test for unpaired observations (two-tailed) was used. The criterion for statistical significance was the probability value $p \leq 0.05$.

Table 1. Body weight of the rats on day 14 after the start of the i.p. treatment of the selective mast-cell secretagogue compound 48/80 for 4 1/2 days which per se suppresses normal body-weight gain moderately and transiently[13]. The animals received a daily s.c. injection of cyclosporine (CS) at different doses and of the saline vehicle for 14 consecutive days. Each group comprised 6 animals. At day 0 the animals weighed 191.8 ± 1.3 g (mean ± SEM.) and there was no significant difference between the groups. The CS treatment significantly hampered the increase in body weight in a dose-dependent manner.

Treatment s.c. days 0–13	i.p. days 0–4	Body weight g mean ± SEM	% change
Saline	48/80	283.4 ± 2.8	(0)
CS 4 mg/kg	48/80	276.4 ± 4.0	− 2
CS 8 mg/kg	48/80	252.0 ± 2.5	− 11 ($p \leq 0.002$)
CS 16 mg/kg	48/80	220.3 ± 7.7	− 22 ($p \leq 0.001$)

Table 2. Blood glucose concentration in rats treated daily with a s.c. injection of cyclosporine (CS) at different doses for 9 days, as well as in s.c. untreated controls. All animals received an i.p. injection of compound 48/80 for 4 1/2 days. The CS treatment caused a dose-dependent hyperglycaemia which was significant in statistical terms at 16 mg/kg.

Treatment s.c. days 0–8	i.p. days 0–4	Blood glucose mmol/l mean ± SEM	% change
Nil	48/80	4.19 ± 0.24(7)	(0)
CS 4 mg/kg	48/80	4.40 ± 0.10(7)	+ 5
CS 8 mg/kg	48/80	4.60 ± 0.23(6)	+ 10
CS 16 mg/kg	48/80	5.51 ± 0.24(6)	+ 32 ($p \leq 0.02$)

Figures in parentheses indicate number of animals.

Table 3. Histamine base concentration in mesenteric windows following a s.c. injection of saline or cyclosporine (CS) in animals which received or did not receive an i.p. injection of the mast-cell secretagogue compound 48/80. There was no difference between the CS and the saline treated animals.

Treatment s.c.	i.p.	Histamine per protein, ng/μg mean ± SEM	% change
Saline	Nil	1.833 ± 0.036(9)	–
Saline	48/80	0.770 ± 0.028(9)	58
CS 4 mg/kg	48/80	0.774 ± 0.038(8)	58

Figures in parentheses indicate number of animals.

Table 4. The effect of s.c. cyclosporine (CS) treatment at a dose of 4 mg/kg/day compared with that of saline on the saline-mediated angiogenic response in the mesenteric windows. Each group comprised 32 windows. CS reduced the mean response by 64% in terms of the number of vessel profiles per unit tissue length and by 28% in terms of the relative vascularized area.

Treatment s.c. days 0–15	i.p. days 0–4	Number of vessels per mm mean ± SEM	Relative vascularized area mean ± SEM
Saline	Saline	1.704 ± 0.470	11.20 ± 4.58
CS	Saline	0.605 ± 0.254 ($p \leq 0.02$)	8.02 ± 2.70 (n.s.)

Results

No animal died during the experimental period and no hemorrhage, ulceration, or any other local tissue reaction was observed at the site of the s.c. CS and saline injections. CS in combination with compound 48/80 reduced the body-weight gain significantly in a dose-dependent fashion (table 1). At a daily dose of 4 mg/kg it exerted no statistically-significant effect, however.

CS increased the blood glucose concentration dose-dependently. At a dose of 16 mg/kg/day, the mean increase was 32%, which was significant in statistical terms (table 2), whereas CS at a dose of 4 mg/kg/day increased the glucose level insignificantly. CS at a dose of 4 mg/kg/day did not influence 48/80-induced mast-cell secretion either (table 3), thereby suggesting that the drug at this dose was non-toxic.

At the apparently atoxic dose of 4 mg/kg/day, CS suppressed saline-mediated-angiogenesis in terms of the number of vessels per unit length (− 64% as a mean; $p \leq 0.02$) and the relative vascularized area (− 28% as a mean; not significant in statistical terms), as shown in table 4. This pattern of effect suggests that CS hampered microvascular branching or tortuosity to a greater extent than it affected the microvascular spatial expansion (see Material and methods).

Discussion

Although anti-angiogenic treatment may soon become a clinical reality, useful in treatment of angiogenesis-dependent diseases such as solid tumours, proliferative retinopathy, arthritis and psoriasis[21], it is sobering to note that all the present candidates for clinical use as

angiogenesis inhibitors appear to exhibit severe host toxicity[22]. More recent studies suggest, however, that there may be a few non-toxic anti-angiogenic agents, such as a synthetic fumagillin analogue[23] and protamine for short periods[24]. Protamine may be immunogenic in humans, however[22]. It is in this perspective that one should see the present finding that CS is an apparently atoxic angiostatic agent in normally-vascularized, adult mammalian tissue. The fact that CS is an established drug certainly increases the potential clinical significance of the present finding.

Clearly, the issue of toxicity should be addressed in great detail in studies of anti-angiogenesis since migrating, proliferating, and differentiating endothelial cells, as well as other cells, growth factors, enzymes and matrix components participating in the angiogenic reaction, are probably vulnerable to any toxic or adverse effects. As there may be differences in the biological effect of the native molecule to be tested and its metabolites formed in vivo, studies of the systemic effect in vivo are also required in most cases to imitate the clinical situation. The clinical use of CS as an immunosuppressant is limited by side-effects such as nephrotoxicity, hepatotoxicity, hypertension, hypertrichosis, gingival overgrowth and a decrease in testicular volume[1,6,25–28]. Although there are a number of similarities between the absorption, distribution and metabolism of CS in humans and in the rat[29], different rat strains, not to mention different species, show a differing tolerance to CS[6,30]. In the present study, a marked angiostatic effect was recorded in Sprague-Dawley rats receiving 4 mg CS/kg/day s.c., which was seemingly atoxic. Daily doses of CS at 7.5–25 mg/kg p.o.[30,31] and of 10 mg/kg s.c.[32] in Sprague-Dawley rats appear not to alter the levels of serum testosterone, serum LH, a number of intratesticular steroid intermediates, serum ceratinine, alkaline phosphatase, SGPT and SGOT. However, Sprague-Dawley rats receiving 10 mg CS/kg/day s.c. may show reduced inulin clearance[29] and bilirubinaemia[32].

In the majority of patients treated daily with CS, psoriatic skin lesions improve considerably and the effect may be visible within a week[4,5,33]. The mechanism of this beneficial effect is not known. It has, however, been claimed that CS exerts a direct anti-proliferative effect on epidermal keratinocytes in culture[34,35] and in xenografts in nude mice[36]. This has been taken as a possible explanation of the beneficial effect of CS in psoriatic skin lesions, where the keratinocytes are hyperproliferative. Other studies refute such an effect at therapeutic doses, or in the presence of serum in the culture medium, however[1,37,38]. It should also be noted that, when the anti-proliferative effect of CS on human keratinocytes was studied in the nude-mouse model[36], a dose of 50 mg CS/kg/day s.c. was used, which is apparently toxic, as was also suggested from the general decline in epithelial proliferation in the host[39].

Since the rat physiologically gains markedly in body weight in adulthood[40], unlike the mouse and most other species used in biomedical research, the effect of drug treatment on body weight gain in rats seems to be a fairly sensitive measure of general toxicity[24]. In the present study, we noted a dose-dependent reduction in body-weight gain as a result of the co-administration of CS and compound 48/80, which was statistically significant at daily doses of 8 and 16 mg/kg. The fact that CS also caused a dose-dependent hyperglycaemia, which was significant in statistical terms at a dose of 16 mg/kg/day, is noteworthy and has, to our knowledge, not been reported previously; hypothetically it relates to β-cell cytotoxicity. CS is, however, not a traditional cytostatic agent since it does not suppress gastrointestinal epithelium or cause myelotoxic, teratogenic, mutagenic or carcinogenic effects[6,26]. In fact, CS can stimulate liver cell proliferation following partial hepatectomy[41] and hair growth, causing hypertrichosis, as well as the proliferation of cultured human fibroblasts[42]. It is, moreover, known that CS in culture media devoid of serum, or containing only small quantities of albumin, can hamper mast-cell secretion in vitro[43–45]. In the present study, however, we found no effect by 4 mg CS/kg s.c. on the histamine secretion of the tissue-bound mast cells in situ in the test tissue in the intact animal, suggesting that the drug at this dose was non-toxic.

It is interesting to note that CS appears to provide protection from the development, growth and spread of certain carcinomas when patients are compared with those receiving conventional immunosuppressive therapy[1,8]. The anti-tumour effect of CS has, moreover, been demonstrated vis-à-vis the rate of spontaneously-occurring tumours in rats treated daily with CS for 2 years[6], and in mouse skin exposed to the tumour-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate, TPA[7]. It is noteworthy that the TPA treatment induces dermal angiogenesis, among other things[46]. CS is also able to enhance the effect of a number of cytotoxic anti-tumour agents in vivo[47,48] by as yet unclear mechanisms. Could the fact that CS a) suppresses the occurrence of spontaneous tumours in normal animals, b) counteracts the neoplastic effect of TPA treatment, c) increases the anti-tumour effect of a number of cytotoxic drugs and, d) exerts beneficial effects in psoriasis, indeed relate to anti-angiogenesis? It should be remembered that the effect of angiostatic therapy may be observed within 24 hours[49,50]. In this context it is noteworthy that CS at low concentrations, even in the presence of serum in the medium, suppresses the replication of cultured rat microvascular and rabbit aortic endothelial cells[51,52] and affects cultured bovine aortic endothelial cells cytotoxically[53]. CS has, in fact, been reported to impair neovascularization, but only at high and apparently toxic doses: at 17 mg/kg/day i.v. in August and Wistar rats, it suppresses revascularization in peripheral nerve grafts, as observed by stereomicroangiography[54].

At 15 mg/kg/day i.p. it decreases vascular ingrowth into transplanted pancreatic islets in mice, recorded by fluorescence microscopy [55].

Clearly, studies are needed to elucidate whether the apparently specific angiogenesis-inhibiting property of CS, which is demonstrated here for the first time, can be exploited in new therapeutic modalities for patients suffering from angiogenesis-dependent diseases. In future investigations on the anti-angiogenicity of CS it will also be interesting to study the structure-activity relationships of analogues which lack immunosuppressive effects [56].

*) The generic name cyclosporine is used in accordance with the recommendation of the United States Adopted Name Council [Borel, J. F., Prog. Allergy *38* (1986) 9].

Acknowledgments. This investigation was supported by the Swedish Medical Research Council, project No. 5942. We are indebted to Mrs Gunvor Jefferth and Miss Ann Nehlmark for their skilful technical assistance.

1 Kanitakis, J., and Thivolet, J., Arch. Derm. *126* (1990) 369.
2 Müller, W., and Graf, U., Schweiz. med. Wschr. *111* (1981) 408.
3 Harper, J. I., Keat, A. C. S., and Staughton, R. C. D., Lancet *2* (1984) 981.
4 Ellis, C. N., Gorsulowsky, D. C., Hamilton, T. A., Billings, J. K., Brown, M. D., Headington, J. T., Cooper, K. D., Baadsgaard, O., Duell, E. A., Annesley, T. M., Turcotte, J. G., and Voorhees, J. J., J. Am. med. Assoc. *256* (1986) 3110.
5 van Joost, T. H., Heule, F., Stolz, E., and Beukers, R., Br. J. Derm. *114* (1986) 615.
6 Ryffel, B., Donatsch, P., Madörin, M., Matter, B. E., Rüttimann, G., Schön, H., Stoll, R., and Wilson, J., Arch. Toxic. *53* (1983) 107.
7 Gschwendt, M., Kittstein, W., and Marks, F., Carcinogenesis *8* (1987) 203.
8 Penn, I., Transplantation *43* (1987) 32.
9 Norrby, K., Jakobsson, A., and Sörbo, J., APMIS *96* (Suppl. 2) (1988) 251.
10 Norrby, K., in: Angiogenesis. On the Quantitative Rat Mesenteric-window Angiogenesis, pp. 282–286. Eds R. Steiner, P. B. Weisz and R. Langer. Birkhäuser Verlag, Basel 1992.
11 Norrby, K., and Sörbo, J., Int. J. exp. Path. *73* (1992) 147.
12 Sörbo, J., and Norrby, K., Agent Actions (1992) Special Conference Issue, C387.
13 Norrby, K., Jakobsson, A., and Sörbo, J., Virchows Arch. [Cell. Path.] *52* (1986) 195.
14 Wassef, R., Cohen, Z., and Langer, B., Transplantation *40* (1985) 489.
15 Norrby, K., Jakobsson, A., and Sörbo, J., Agent Actions *30* (1990) 231.
16 Norrby, K., Jakobsson, A., and Sörbo, J., Microvasc. Res. *39* (1990) 341.
17 Norrby, K., Jakobsson, A., Simonsen, M., and Sörbo, J., Experientia *46* (1990) 856.
18 Norrby, K., Jakobsson, A., and Sörbo, J., Virchows Arch. [Cell. Path.] *57* (1989) 251.
19 Werner, W., Rey, H.-G., and Wielinger, H., Z. analyt. Chem. *252* (1970) 224.
20 Norrby, K., Bergström, S., Druvefors, P., APMIS *91* (1983) 195.
21 Editorial., Lancet *337* (1991) 208.
22 Maione, T. E., and Sharpe, R. J., TIPS *11* (1990) 457.
23 Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H., and Folkman, J., Nature *348* (1990) 555.
24 Jakobsson, A., Sörbo, J., and Norrby, K., Int. J. exp. Path. *71* (1990) 909.
25 Ptachcinski, R. J., Burckart, G. J., and Venkataramanan, R., Drug Intell. Clin. Pharm. *19* (1985) 90.
26 Bennett, W. M., and Norman, D. J., A. Rev. Med. *37* (1986) 215.
27 Sikka, S. C., Koyle, M. A., Swerdloff, R. S., and Rajfer, J., Transplantation *45* (1988) 784.
28 Sikka, S. C., Bhasin, S., Coy, D. C., Koyle, M. A., Swerdloff, R. S., and Rajfer, J., Endocrinology *123* (1988) 1069.
29 Sullivan, B. A., Hak, L. J., and Finn, W. F., Transpl. Proc. *17* (Suppl. 1) (1985) 145.
30 Whiting, P. H., Thomson, A. W., and Simpson, J. G., Transpl. Proc. *17* (Suppl. 1) (1985) 134.
31 Rajfer, J., Sikka, S. C., Lemmi, C., and Koyle, M. A., Endocrinology *121* (1987) 586.
32 Seethalakshmi, L., Menon, M., Malhotra, R. K., and Diamond, D. A., J. Urol., part 2, *138* (1987) 991.
33 Meuller, W., and Herrmann, B., N. Engl. J. Med. *301* (1979) 555.
34 Furue, M., Gaspari, A. A., and Katz, S. I., J. Invest. Derm. *90* (1988) 796.
35 Nickoloff, B. J., Fisher, G. J., Mitra, R. S., and Voorhees, J. J., Am. J. Path. *131* (1988) 12.
36 Urabe, A., Kanitakis, J., Viac, J., and Thivolet, J., J. Invest. Derm. *92* (1989) 755.
37 Kato, N., Halprin, K. M., and Taylor, J. R., J. Invest. Derm. *88* (1987) 52.
38 Fisher, G. J., Duell, E. A., Nickoloff, B. J., Annesley, T. M., Kowalke, J. K., Ellis, C. N., and Voorhees, J. J., J. Invest. Derm. *91* (1988) 142.
39 Ramirez-Bosca, A., Kanitakis, J., and Thivolet, J., Cancer *66* (1990) 936.
40 Rogers, A. E., in: The Laboratory Rat, vol. I. Nutrition, pp. 123–128. Eds H. J. Baker, J. R. Lindsey and S. H. Weisbroth. Academic Press, New York.
41 Kim, Y. I., and Calne, R. Y., Surg. Gynec. Obstet. *166* (1988) 317.
42 Bartold, P. M., J. Periodont. Res. *24* (1989) 314.
43 Triggiani, M., Cirillo, R., Lichtenstein, L. M., and Marone, G., Int. Arch. Allergy appl. Immunol. *88* (1989) 253.
44 Dráberová, L., Eur. J. Immunol. *20* (1990) 1469.
45 Ezeamuzie, I. C., and Assem, E. S. K., Agents Actions *30* (1990) 110.
46 Aldaz, C. M., Conti, C. J., Gimenez, I. B., Slaga, T. J., and Klein-Szanto, J. P., Cancer Res. *45* (1985) 2753.
47 Meador, J., Sweet, P., Stupecky, M., Wetzel, M., Murray, S., Gupta, S., and Slater, L., Cancer Res. *47* (1987) 6216.
48 Twentyman, P. R., Anticancer Res. *8* (1988) 985.
49 Sidky, Y. A., and Borden, E. C., Cancer Res. *47* (1987) 5155.
50 Hill, S., Williams, K. B., and Denekamp, J., Eur. J. Cancer clin. Oncol. *25* (1989) 1419.
51 Lau, D. C. W., and Wong, K. L., Transpl. Proc. *19* (1987) 3496.
52 Ferns, G., Reidy, M., and Ross, R., Am. J. Path. *137* (1990) 403.
53 Zoja, C., Furci, L., Ghilardi, F., Zilio, P., Benigni, A., and Remuzzi, G., Lab. Invest. *55* (1986) 455.
54 Grochowicz, P., Schätzle, M., Hammer, C., Olscewski, W., and Brendel, W., Handchirurgie *17* (1985) 17.
55 Rooth, P., Dawidson, I., Lafferty, K., Diller, K., Armstrong, J., Pratt, P., Simonsen, R., and Täljedal, I.-B., Diabetes *38* (Suppl. 1) (1989) 202.
56 von Wartburg, A., and Traber, R., Prog. Allergy *38* (1986) 28.

0014-4754/92/11-12/1135-04$1.50 + 0.20/0