Br. J. Pharmac. (1978), **64**, 123–128.

CHANGES IN BLOOD FLOW AND MEDIATOR CONTENT OF RABBIT SKIN GRAFTS

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1 Blood flow changes have been measured in rabbit skin homografts and autografts and an attempt has been made to correlate these changes with the presence of vasoactive agents in homogenates of the grafts.

2 During the healing-in of both types of grafts the blood flow and histamine content increased.

3 If this histamine was responsible for the vasodilatation via an H_2-receptor it must have been released in the form of 'nascent' or intrinsic histamine since the vasodilatation was not antagonized by antihistamine.

4 A peak of histamine concentration occurred in homografts, more than three times that in autografts at the onset of rejection. At the same time the blood flow stopped completely.

5 This vasoconstriction might be mediated by histamine since treatment with mepyramine delayed the cessation of blood flow from 5 days up to 7 to 10 days.

6 Prostaglandins appeared to be involved only in the period of peak blood flow in homografts because indomethacin did not delay the onset of rejection, but reduced the peak blood flow in the homografts at a time when there was an increased content of prostaglandin E_2 in the homograft tissue.

Introduction

When skin is transplanted as either autografts or homografts, distinct vascular changes occur during healing-in and in the case of the homograft also at the onset of rejection (Medawar, 1944). The question arises, do these vascular changes reflect the activity of pharmacological mediators? To investigate this possibility, an attempt was made to correlate the blood flow changes which occur in grafted skin with the presence of mediators in homogenates of the graft tissue.

Methods

Skin graft procedure

Adult New Zealand (NZ) and Norfolk White (NF) rabbits weighing 3.0 to 4.0 kg anaesthetized with sodium pentobarbitone (40 mg/kg i.v.) were used. Full thickness skin grafts (2.0 × 2.0 cm) were removed from the hind limbs and either transplanted onto the hind limbs or onto the back from which similar size patches of dorsal skin had been removed (Jasani & Lewis, 1971). For the homografts the NZ and NF rabbits were paired and both strains were used for the autografts.

Collection, homogenization and extraction of tissues

For the estimation of mediators in the grafts, rabbits received either 12 homografts of leg skin transplanted onto the back, or 5 autografts of leg skin transplanted onto the legs. At various times after grafting, the sutures were carefully removed and the graft was torn from the graft bed with a sturdy pair of toothed forceps and instantly frozen in alcohol and dry ice. Pressure was maintained on the graft bed with a swab until bleeding had stopped (usually 2 to 3 min). The removed grafts were blotted dry, weighed and wrapped in tin foil and stored at −20°C until they were extracted. Non-operated skin was removed to provide controls and treated in the same manner as the grafts. For homogenization the tissues were thawed, finely chopped with scissors and suspended in 2 ml of ice-cold 60% ethanol, and homogenized at 4°C with a Polytron Ultra Turax homogenizer run at 30% of the maximum speed for 90 seconds. The homogenizer was washed and the washings added to

the homogenate, with a further 2 ml of ice-cold 60% ethanol. The homogenate was centrifuged at 5,000 rev/min for 30 min to obtain a clear supernatant. The supernatant was poured into a 25 ml evaporating flask and the pellet was resuspended in 3 ml of 60% ethanol and mixed on a vortex rotor for 60 s and centrifuged. The supernatants were pooled and the pellet was discarded.

The extract was evaporated to dryness under a partial vacuum at 37°C in a Rotavapor-R (Buchi). The dried extract was dissolved in 4 ml of saline (0.9% w/v NaCl solution) and divided into 1 ml aliquots in plastic tubes, capped and stored at −20°C until they were assayed. These samples were assayed direct, with no further purification except for the prostaglandin assay. Some samples were further extracted for prostaglandins (Gilmore, Vane & Wyllie, 1968) to check that the protein in the samples did not affect the results of the radioimmunoassay.

Assay of mediators

Each tissue extract was assayed for prostaglandins E_2 and $F_{2\alpha}$, histamine and 5-hydroxytryptamine (5-HT). A radioimmunoassay was used for the prostaglandins (Hennam, Johnson, Newton & Collins, 1974). Before the histamine and 5-HT contents were assayed, the protein in each sample was precipitated with perchloric acid and the supernatant was assayed by an automated fluorimetric technique (Bogdanski, Pletscher, Brodie & Udenfriend, 1956; Shore, Burkhalter & Cohen, 1959; Maickel & Miller, 1966; Evans, Lewis & Thomson, 1973).

Blood flow measurement

For all blood flow measurements, the skin grafts were transplanted from the legs onto the back, each animal received 12 grafts and both autografts and homografts were studied.

Each rabbit was restrained in a box, and a micro injection of ^{133}Xenon in saline (0.01 ml) was given intradermally to normal skin or grafted tissue. The washout of radioactivity was monitored for at least 30 min with a collimated γ-scintillation detector (Lewis, Peck, Williams & Young, 1976). The detector, a sodium iodide thalium activated crystal was placed directly above the site of injection, about 1 cm from the surface of the rabbit. The washout was recorded every 40 s and printed automatically on a Panax recorder. The ^{133}Xe was diluted according to its age to give an initial count of at least 35,000 but not greater than 80,000 counts in the first 40 s period. The results were expressed as a percentage of the initial count and plotted on a semi-logarithmic scale against time. The curve peeling technique was used to draw two exponentials from the curve, and the half times ($T_{1/2}$) for ^{133}Xe in the skin and in the subcutaneous tissue were calculated (Sejrsen, 1969). From these half times the clearance constant, K was calculated giving an indirect measurement of blood flow in the tissue. All blood flows will be expressed in terms of K.

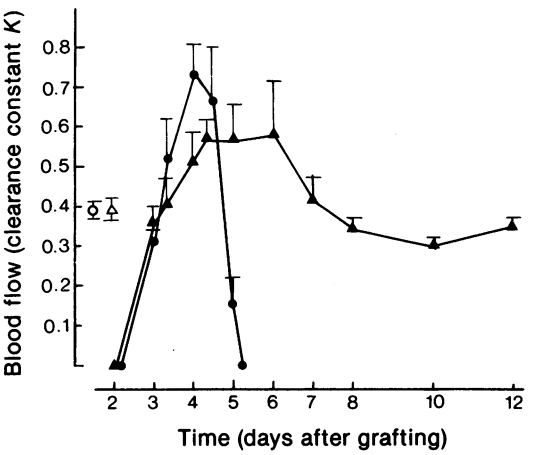

Figure 1 Blood flow changes in rabbit skin autografts (▲) and homografts (●). The blood flow is expressed in terms of the clearance constant *(K)* for Xenon,

$$K = \frac{0.6932}{T_{1/2}}.$$

Each point represents the mean of 4 experiments for autografts, 7 experiments for homografts; vertical lines show s.e. means. No blood flow could be detected before day 3, at which time it was similar to that of normal skin (○ △). In the autograft, the blood flow increased forming a plateau between day 4 and 6, and subsequently decreased to normal skin blood flow. In the homograft the plateau of high blood flow was maintained for 12 h only, after which no further blood flow could be detected.

The effect of several pharmacological antagonists on the blood flow of both the homograft and the autograft was measured by the ^{133}Xenon clearance method. The following antagonists were given intravenously in divided doses from the first or third day after grafting: indomethacin (2×5 and 10 mg kg^{-1} day^{-1}), methysergide (3×0.3 mg kg^{-1} day^{-1}), mepyramine (3×2.5 mg kg^{-1} day^{-1}), metiamide (3×50 mg kg^{-1} day^{-1}), and metiamide plus mepyramine ($3 \times 50 + 2.5$ mg kg^{-1} day^{-1}).

Results

Blood flow changes

The blood flow measurements were made in animals in which 12 leg skin autografts or homografts had been transplanted onto the back. The blood flow

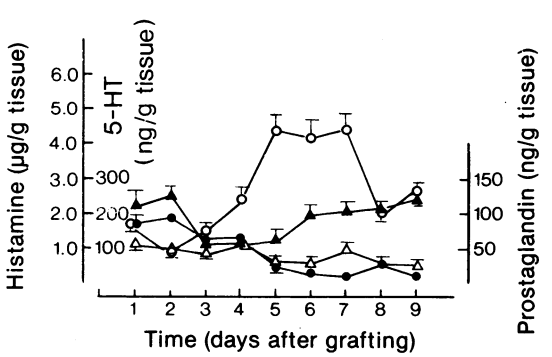

Figure 2 Mediator content of rabbit skin autografts. A total of 5 grafts were transplanted onto each animal. Each point represents the mean of at least 8 samples; vertical lines show s.e. means. There was no significant change in the tissue content of prostaglandin E_2 (PGE_2, ●), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, △), and 5-hydroxytryptamine (5-HT, ▲) all expressed as ng/g tissue. However, the histamine content as μg/g tissue (○) increased significantly at day 3–4 and formed a plateau between day 5 and 7, after which the histamine content decreased to that of normal skin. The content of control skin was histamine 2.3 ± 0.4 μg/g, 5-HT 112 ± 10 ng/g, PGE_2 13 ± 1.6 ng/g, $PGF_{2\alpha}$ 10 ± 1.5 ng/g.

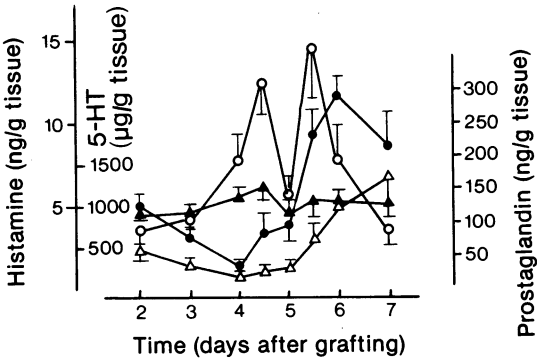

Figure 3 Mediator content of rabbit skin homografts. All animals each received a total of 12 grafts. Each point represents the mean of 4 samples; vertical lines show s.e. means. The histamine content as μg/g tissue (○) formed 2 peaks, the first coincided with a significant short-lived plateau of prostaglandin E_2 (PGE_2) as ng/g tissue (●). The second occurred together with increases in PGE_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, △) after day 5 i.e. after rejection of the homograft. There was no significant change in the 5-hydroxytryptamine (5-HT) content (▲). The content of control skin was histamine 2.1 ± 0.23 μg/g, 5-HT 1125 ± 181 ng/g, PGE_2 28 ± 3.5 ng/g, $PGF_{2\alpha}$ 15 ± 3 ng/g.

expressed in terms of the clearance constant for ^{133}Xenon is plotted against days after grafting in Figure 1. No blood flow was detected in either the autografts or the homografts until day 3 at which time it was similar to that of normal skin. Between days 3 and 4 it increased rapidly reaching a peak around day 4 in both autografts and homografts. In autografts the peak flow was maintained until day 6, after which the flow gradually returned to normal during the following 4 to 6 days. In the homografts, the peak flow was maintained for only a 12 h period. This was followed by a sudden fall in flow and at 5 days, 6 h the flow had ceased altogether.

Mediator content of autografts

At daily intervals up to 9 days after grafting all grafts were removed from one animal at the same time and processed separately. The content of the different mediators in the autografts is illustrated in Figure 2. During the healing-in of the autograft over the first 4 days after grafting, the contents of prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$ and 5-HT levelled off to values found in normal skin and remained within those levels up to day 9. The histamine content, however, increased significantly on day 4 and continued to increase on day 5 (4.4 ± 0.48 μg/g tissue) and remained at this level until day 7. On day 8 the histamine content fell to 2.1 ± 0.18 μg/g tissue which was similar to values found in normal skin.

Mediator content of homografts

Homografts of leg skin transplanted onto the backs of 4 rabbits were removed individually at intervals from day 2 to day 7 after grafting. The different mediator contents assayed in the homograft tissue are illustrated in Figure 3. As with the autografts, during the initial healing-in period, the contents of all mediators were similar to those of normal skin except for histamine. There was no change in the 5-HT content throughout the course of the experiment. The histamine content reached two peaks, one on day 4, hour 12 (12.5 ± 2.5 μg/g tissue) and the second on day 5, hour 12 (14.5 ± 4.3 μg/g tissue) i.e. after the blood flow had stopped completely. Between these peaks, on day 5, the histamine content fell significantly to 5.8 ± 1.4 μg/g tissue. By day 6 the histamine content had decreased and continued to do so up to day 7 (3.3 ± 1.1 μg/g tissue).

The changes in prostaglandin content produced a different picture from that of the histamine content. The PGE_2 remained low up to day 4 but between day 4, hour 12 and day 5 the content increased (94.7 ± 27.1 ng/g tissue), forming a short-lived plateau which coincided with the peak of blood flow. After

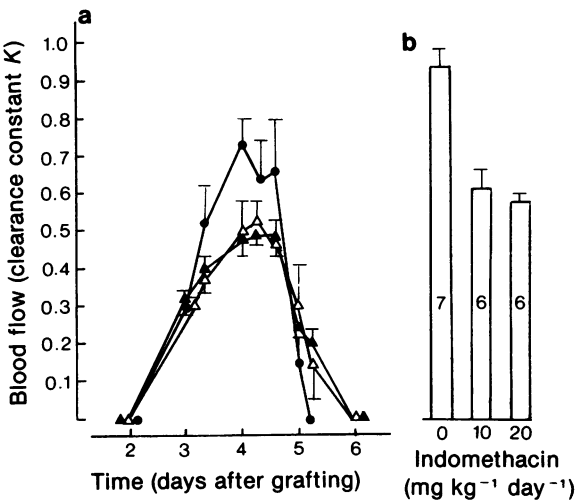

Figure 4 The effect of indomethacin on the blood flow changes in rabbit skin homografts. Blood flow is expressed as in Figure 1. Two groups of animals received indomethacin 2 × 5 mg kg^{-1} day^{-1} (△) and 2 × 10 mg kg^{-1} day^{-1} (▲) given intravenously from day 3 after grafting. Each point represents the mean; vertical lines show s.e. means. The homografts on animals treated with indomethacin failed to reach the maximum blood flow of the untreated homografts (●). This is more clearly illustrated in (b) by the histograms which represent the mean of the peak flow attained in each animal. The effect of indomethacin was to decrease the maximum blood flow by 35–40%. The numbers in the histograms refer to the number of animals.

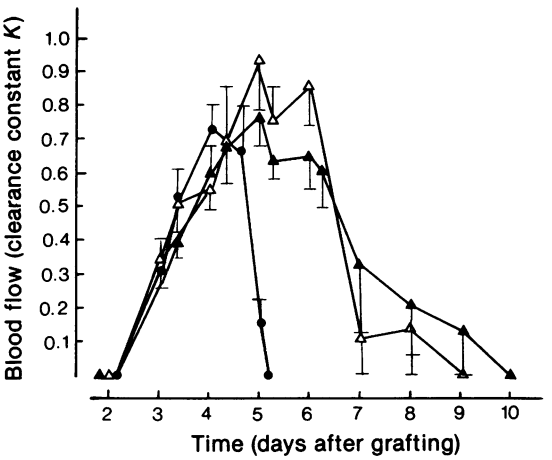

Figure 5 The effect of mepyramine on the blood flow changes in rabbit skin homografts. Blood flow is expressed as in Figure 1. Two groups of animals received mepyramine 3 × 2.5 mg kg^{-1} day^{-1}, group I (▲) from day 3 and group II (△) from day 1 after grafting until rejection had occurred. The cessation of blood flow of the homografts of the mepyramine-treated animals occurred 7 to 10 days after grafting compared with day 5 in the untreated homografts (●). Each point represents the mean of results from 4 to 7 animals; vertical lines show s.e. means.

the blood flow in the homograft had stopped there was a further increase to very high levels up to day 7 (215.1 ± 57.0 ng/g tissue) after grafting. At the time of the main increase in PGE_2 content there was also an increase in $PGF_{2\alpha}$ content. On day 5, hour 12 this reached 76.2 ± 23.6 ng/g tissue.

Effect of antagonists on the blood flow

In a series of experiments in which animals receiving autografts were treated with indomethacin, mepyramine, metiamide or methysergide before grafting, there was no influence on the blood flow pattern established by the autografts.

On the other hand, both indomethacin and mepyramine affected the blood flow changes during the homograft reaction. Two groups of 6 animals were given indomethacin (5 and 10 mg/kg) intravenously twice a day from day 3 after grafting. In neither group did the blood flow reach the maximum blood flow found in the untreated homografts (Figure 4). The effect of indomethacin on the maximum blood flow achieved by the individual homografts is further illustrated in Figure 4b. Although indomethacin affected the maximum blood flow, it did not alter the time of rejection and hence the survival of the homograft.

While metiamide (3 × 50 mg kg^{-1} day^{-1}) (H_2-receptor antagonist) did not alter the blood flow pattern, mepyramine (H_1-receptor antagonist) prolonged the increased blood flow and increased the homograft survival time from 5 days to 7 to 10 days (Figure 5). Two groups of animals were treated with mepyramine (3 × 2.5 mg kg^{-1} day^{-1}) group I (5 animals) from day 3 and group II (4 animals) from day 1 after grafting until rejection had occurred. The time at which mepyramine treatment began did not significantly alter the effect on the blood flow of the homograft.

When three animals were treated with a combination of mepyramine and metiamide from day 1, the peak of high blood flow in the homograft was maintained for about 36 h instead of 12 h in untreated animals before rejection occurred.

Two groups of animals received methysergide (5-HT antagonist) either from day 1 or day 3 after grafting until rejection had occurred. Methysergide like metiamide, did not alter the blood flow pattern established by the homograft.

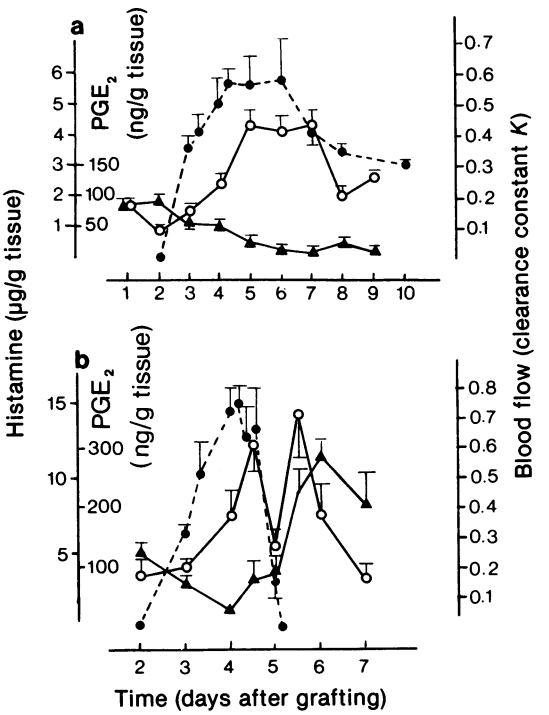

Figure 6 Comparison of blood flow changes (●) with the content of histamine (○) and prostaglandin E_2 (PGE_2, ▲) in the rabbit skin autograft (a) and homograft (b). Data were obtained from Figures 1, 2 and 3; blood flow is expressed as in Figure 1. There is a good correlation between the blood flow changes and the histamine content during the healing-in process in both types of grafts. The peak flow in homografts was accompanied by a small significant increase in PGE_2 content. The cessation of blood flow in homografts occurs at a time when the histamine content was maximal. The secondary increase in the histamine and PGE_2 contents occurred after rejection of the homograft i.e. cessation of blood flow. Each point represents the mean; vertical lines show s.e. means.

Discussion

The purpose of this study was to investigate the possible contribution made by pharmacological mediators to the changes of blood flow which occur in skin autografts and homografts. Although the changes have been well documented by visual observations, in the present study ^{133}Xe clearance has been used as a more precise measure of blood flow. When transplanted skin becomes revascularised there is a pronounced vasodilatation in the tissue and in the case of the homograft vasoconstriction occurs at the onset of rejection. In the present experiments the increased vasodilatation was maintained for several days in the autografts until the flow returned to that of the normal surrounding skin. In homografts the peak flow was maintained for only about 12 h until the onset of rejection when the blood flow ceased.

Increase in blood flow during the healing-in of both autografts and homografts was accompanied by an increase in tissue histamine (see Figure 6). However, if the histamine present in the tissue is responsible for producing this vasodilatation, it would appear to be released in a form that is not accessible to antagonists since neither H_1- nor H_2-histamine receptor antagonists, when given systemically throughout the experiment, altered the blood flow changes during the healing-in process. The taking of a graft involves growth of new tissue and is similar to the tissue repair process studied by other workers (Kahlson, 1962; Moore & Schayer, 1969). Kahlson and his colleagues (1962) in studying the process of tissue growth have suggested that histamine is formed by the action of the enzyme histidine decarboxylase as 'nascent' histamine which is not antagonized by antihistamines. Schayer (1962) has shown that this enzyme has an inducible activity and he has proposed an important role for histamine in microcirculatory regulation. The induced histamine may be synthesized in or near vascular endothelial cells and may be intrinsic according to the definition of Dale (1948).

In the present experiments, therefore, the tissue histamine found during the healing-in of autografts and homografts represent 'nascent' or intrinsic histamine, which would explain the resistance of the vascular effect to antihistamine agents. However, as tissue homogenates were used for extraction of the mediators, it was not possible to distinguish between bound and free histamine. Therefore, during the healing-in of the grafts it is possible that the histamine formed remained bound or intracellular so that it was not functional and therefore not responsible for the concomitant vasodilatation.

Both H_1- and H_2-histamine receptors have been shown to exist in the peripheral vascular system of the rabbit (Parsons & Owen, 1973; Brimblecombe, Owen & Parsons, 1974). These workers have suggested that the normal response to histamine reflects the balance between the opposing vasoconstrictor (H_1-receptors) and the vasodilator (H_2-receptors) effects. This balance changes under differing conditions. The vasodilator phase usually predominates when smaller doses of histamine are given while larger doses of histamine give rise to prolonged vasoconstriction.

The histamine content of the homograft at its highest i.e. just before cessation of blood flow (see Figure 6) was at least 3 times greater than that in the autograft and more than 6 times that in control skin. It seems possible that whereas the concentration of hist-

amine present during the healing-in process was only sufficient to cause vasodilatation, at its peak concentration the histamine caused vasoconstriction sufficient to bring on the onset of rejection of the tissue.

Unlike the histamine present during the early stages of the reaction, the histamine present during the peak concentration appeared to act on H_1-receptors to cause the vasoconstriction because in animals treated with the antagonist, mepyramine, the vasoconstriction was overcome and the onset of rejection was delayed from 5 days up to 7 to 10 days. The animals were treated with the antihistamine throughout the experiment until rejection occurred. In spite of this, delayed rejection still occurred, accompanied by cessation of blood flow. It is not clear at present whether sufficient histamine was generated to overcome the effect of mepyramine or some other vasoconstrictor mechanism was involved.

Henney, Bourne & Lichtenstein (1972) suggested that histamine has the ability to suppress the cytolytic activity of T-lymphocytes and that this action is mediated via an H_2-receptor. It is possible that mepyramine being an H_1-antagonist acts by diverting the limited amount of histamine available preferentially to H_2-receptor sites on the sensitized lymphocytes, and consequently delaying rejection. However, it was not possible to test this view by the use of the H_2-receptor antagonists in the present investigation, since the rejection reaction occurred at the maximum intensity and any potentiation that might have occurred with H_2-receptor antagonists would not have been evident. However, it was evident that the delayed rejection caused by mepyramine was partly reversed by metiamide.

Since indomethacin, a potent inhibitor of prostaglandin formation, did not delay the onset of rejection, it appears that prostaglandins do not play a role in this part of the reaction. However, it might well be that a prostaglandin of the E series, probably E_2, is partly responsible for the peak increase of blood flow in homografts. Firstly, at this time there was a small but significant increase in the PGE_2 content of the homografts but not the autografts. Secondly, the homografts on animals treated with indomethacin all failed to reach the peak blood flow of the untreated homografts; this was reduced by 35–40%. The peak blood flow in autografts was unaffected by indomethacin.

This work was supported by the MRC, the Vandervell Foundation and the Smith & Nephew Foundation.

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(*Received February 28, 1978. Revised April 12, 1978.*)