Calcitonin gene-related peptide improves skin flap survival and tissue inflammation

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Summary The effects of systemic administration of calcitonin gene-related peptide (CGRP) on survival and inflammation of experimental skin flaps subjected to prolonged arterial ischemia were studied. An island groin flap was elevated in the rat. The femoral artery was occluded for 8, 10, 12 or 14 h in four groups of 10 rats. In a group of 10 sham-operated control animals, the femoral artery was not occluded. After ischemia, blood flow was restored and flap survival evaluated at day 7. Following 12 h of ischemia, three flaps (30%) survived, compared with 100% survival of the control group. In the second part of the study the effects of CGRP on flap survival were assessed. Eighty flaps were rendered ischemic for 12 h, and received systemic CGRP (10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M) or saline (control) at the end of the ischemia period. Administration of CGRP (10^{-7} M) significantly increased the number of flaps surviving compared with the control. The effect of systemic pretreatment of the animals with the CGRP receptor antagonist CGRP8-37, followed by CGRP (10^{-7} M) treatment was also evaluated in 10 flaps. Flap survival in this group was 10%. In the third part of the study the anti-inflammatory effects of CGRP were evaluated. Forty rats were subjected to arterial ischemia for 12 h, and received systemic CGRP (10^{-7} M), or saline at the end of the period of ischemia. The animals were sacrificed at 24 h and flap tissue samples were obtained. Myeloperoxidase (MPO) analysis was used as marker of neutrophil accumulation. CGRP (10^{-7} M) significantly reduced the 24 h MPO accumulation in the flap, compared with saline treatment. A group of animals was pretreated with CGRP8-37, followed by CGRP (10^{-7} M), and a significant increase of MPO accumulation was seen, compared with the group treated only with CGRP. This study suggests that CGRP has a beneficial effect on survival of the rat ischemic groin flap, and diminishes the inflammatory response to the ischemic insult.

INTRODUCTION

Soft tissue defects resulting from cancer and injury often necessitate the use of pedicled flaps or free tissue transfers. These procedures may be complicated by partial or even complete flap loss, despite numerous clinical and experimental efforts to reduce tissue necrosis. One reason for the recurring problems is the decrease in tissue perfusion secondary to vasoconstriction. The mechanism for vasoconstriction is not completely understood, but vessel spasm and endothelial damage play an important role. Another mechanism with potential to damage flap tissue is inflammatory recruitment of leukocytes, a well-established feature of physical tissue injury. Phagocyte recruitment during inflammation is a host defence against pathogen invasion; however, the microbicidal toxins (e.g. oxidants and proteolytic enzymes) released from granulocytes may be destructive to host tissues as well. Neutrophil accumulation has been shown to be associated with flap surgery, and the injury resulting from ischemia–reperfusion is known to be a powerful stimulus for leukocyte recruitment. The role of
neutrophils in flap necrosis has been demonstrated in experimental skin flaps. Earlier studies showed that pre-treatment with certain anti-inflammatory agents (e.g. leukotriene synthesis inhibitors, oxygen radical scavengers) may improve skin flap survival. Several pharmacological methods have been employed to relieve experimental and clinical flaps, with varying results. Local anesthetics, particularly lidocaine are used intraoperatively as spasmolytic agents. Calcitonin gene-related peptide (CGRP) is an endogenous substance regarded as one of the most potent vasodilators isolated. The vascular properties of CGRP have been the subject of numerous investigations. CGRP increases blood flux in ischemic tissue, and the microcirculation of experimental and clinical flaps. CGRP has also been shown to exert anti-inflammatory effects, and is thought to be involved in the adaptive response to ischemia. The purpose of this study was to examine if intravenous (i.v.) CGRP treatment improves survival and reduces neutrophil accumulation in surgical flaps in the rat.

**MATERIALS AND METHODS**

The study involved three phases. In the first phase, 50 male albino rats (Sprague-Dawley, body weight 150–200 g) were anesthetized with chloral hydrate 0.4 g/kg, ip (Kebo, Sweden) and the groins were depilatated. The rats were placed on a heating pad with a constant temperature of 38°C and a 2.5 cm diameter adipocutaneous neurovascular island flap based on the superficial epigastric blood vessels was raised. The feeding femoral artery was dissected under a Zeiss operating microscope (Opmi 1 FC) and occluded with two microclamps (proximal and distal to the epigastric artery) (Fig. 1). To establish the critical time for flap survival, the animals were divided into four groups of 10, and the femoral artery remained occluded in each group for 8, 10, 12 or 14 h. In a group of 10 sham-operated control animals, the femoral artery was not occluded. After the period of ischemia, the microclamps were removed, the flaps restored to their anatomical bed, and the skin was closed with a 4–0 nylon suture. Seven days after surgery, the animals were killed with an overdose of pentobarbital (120 mg/kg i.p.), and the survival of the flap was estimated visually and by palpation. Necrotic flaps were reproducibly indurated and pale, while surviving flaps were indistinguishable from the surrounding tissue.

In the second part of the study, the effects of CGRP on flap survival were evaluated: 90 groin flaps were raised as described, and rendered ischemic for 12 h. CGRP (0.5 ml; 10⁻², 10⁻⁴, 10⁻⁶, 10⁻¹⁰ M, Peninsula Laboratories,
Merseyside, UK) was given i.v. in the contralateral femoral vein (n = 10 in each CGRP group), or saline (n = 10) was administered systemically, before the end of the period of ischemia. In another group of 10 flaps, pretreatment with the CGRP receptor antagonist CGRPS\textsubscript{37} (Peninsula Laboratories) was performed (30 pmol, given i.v. in the contralateral femoral vein immediately before surgery). This was followed by treatment with CGRP (10\textsuperscript{-7} M) at the end of the surgical procedure, as described above. The flaps were again restored to their anatomical bed, and their survival evaluated on day seven, as described earlier.

In the third part of the study the anti-inflammatory effects of CGRP were evaluated, in 40 groin flaps subjected to arterial ischemia for 12 h. The animals received 0.5 ml of CGRP (10\textsuperscript{-7} M) (n = 15) given i.v. in the contralateral femoral vein, CGRPS\textsubscript{37} (30 pmol, given i.v. in the contralateral femoral vein immediately before surgery, n = 10) followed by CGRP (10\textsuperscript{-7} M) treatment, or saline (n = 15). At the end of the period of ischemia, the flaps were restored to their anatomical bed. The animals were sacrificed at 24 h. The flaps were removed, weighed, homogenised in 10 ml 0.5% hexadecyltrimethylammonium bromide, and freeze-thawed, then the myeloperoxidase (MPO) activity of the supernate was assessed. The enzyme activity was determined spectrophotometrically by the change in absorbency at 650 nm (25°C) occurring in the redox reaction of H\textsubscript{2}O\textsubscript{2} tetramethylbenzidine catalysed by MPO. Values are expressed as MPO units/g tissue.

All solutions of drugs and vehicle were kept coded until flap survival had been evaluated at the end of the experiments.

### Statistical analysis

Two-tailed tests were used for statistical analysis. Analysis of paired observations was performed by the Fisher's exact test, MPO data were compared by the Student's t test. P values < 0.05 were considered significant. All values are expressed as mean ± SD. Unless stated otherwise, n represents number of animals per group.

### RESULTS

In a first series of experiments, flap survival was inversely related to the duration of femoral artery occlusion: six flaps survived after 8 h, five flaps survived after 10 h and only three after 12 h. One flap survived after 14 h. A significant difference in flap survival was seen when comparing the 10, 12 and 14 h periods of occlusion with the control group (100% survival, P < 0.05) (Table 1). Because 12 h of occlusion was found to reliably cause the demise of a significant number of flaps, this period of time was arbitrarily chosen to examine the effects of CGRP on flap survival.

In the second set of experiments, CGRP (10\textsuperscript{-7} M) significantly increased the number of flaps surviving compared with the control (9 versus 2, P < 0.05). Other CGRP concentrations did not significantly change flap survival. In the group pretreated with CGRPS\textsubscript{37}, only one flap survived (P < 0.005, compared with the group treated with CGRP alone) (Table 2).

In the third group of experiments, the saline-treated rats averaged a flap MPO concentration of 37.5 ± 7 units/g tissue, 24 h after surgery. CGRP (10\textsuperscript{-7} M) significantly reduced the MPO accumulation in the flap (23.3 ± 1.2, P < 0.0005). A significant increase of MPO accumulation was seen in the CGRPS\textsubscript{37} pretreated group, compared with the group treated only with CGRP (mean MPO in CGRPS\textsubscript{37} group was 51.5 versus 23.3 units/g tissue, P < 0.0005) (Table 3).

### DISCUSSION

In this study, the systemic administration of low CGRP concentrations significantly improved the survival of surgical flaps in the rat after a prolonged period of ischemia.

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This effect of CGRP was dose-dependent, and it required only a single administration in order to be effective. These findings are consistent with the observations of Kjartansson and Dalsgaard,¹⁴ who used a random dorsal skin flap model in the rat to show that low doses of CGRP injected locally into the venous circulation of the flap caused a similar improvement in tissue survival. The model of action by which such low doses of CGRP increase flap survival is still unknown. One possibility is that CGRP increases blood flow in the flap following an ischemic insult by causing vasodilatation. Indeed, CGRP is currently regarded as one of the most potent vasodilators isolated. The release of CGRP from sensory nerve fibers is increased during ischemia, and this has been suggested to enhance tissue repair and be involved in the adaptation to ischemia.²⁰ Clinically the survival of ischemic surgical flaps is increased after treatment of the flap with CGRP.¹²,¹³,¹⁷ Conversely it is significantly reduced after CGRP is depleted by capsaicin pretreatment.¹⁵ CGRP acts as a local factor stimulating endothelial cell proliferation, and is important in the formation of new vessels.²⁰ In vitro studies have shown that concentrations of 10⁻¹⁰⁻¹⁰⁻⁷ M of CGRP are capable of relaxing human radial arteries and saphenous veins and porcine aortas.¹⁴,¹⁸,²¹ CGRP also increases the blood flow in ischemic tissue, promoting the survival of experimental and clinical flaps.¹⁶,¹⁷ In healthy volunteers CGRP given intravenously and intra-arterially increased blood flow.¹⁴ In a recent study CGRP was found to improve healing of venous stasis ulcers in humans, probably by improving the tissue perfusion and stimulating fibroblast proliferation (unpublished observations). In the present model, the beneficial effect of CGRP on flap survival was not seen when the animals were pretreated with the CGRP receptor antagonist CGRP₈−₃₇, demonstrating that CGRP was responsible for the improved flap survival observed. CGRP₈−₃₇ pretreatment has been shown to increase 5-hydroxytryptophan-induced edema formation in the rat hindpaw, thus blocking the anti-inflammatory action of endogenously released CGRP.¹⁹

An alternative mechanism of action by which CGRP may act is the inhibition of the inflammatory process and leukocyte recruitment, a well-known feature of mechanical trauma with potential to cause further tissue injury.²⁴ The enzyme myeloperoxidase is abundant in neutrophils¹ and has been found to be a reliable marker for the detection of neutrophil accumulation in inflamed skin in vivo.²² The present study found that CGRP treatment markedly reduced the accumulation of MPO in the flap after prolonged ischemia. While some investigators have suggested that CGRP may be an important proinflammatory mediator,²³ the finding in the present study that low concentrations of CGRP inhibited dermal neutrophil recruitment, as measured by MPO concentration, indicates that CGRP may also act to control inflammation.

Pretreatment with CGRP₈−₃₇ resulted in a significant increase of MPO concentration in the flap if compared with the group treated with CGRP or saline. Previous studies have demonstrated that CGRP can inhibit inflammatory edema formation in several different species,¹²,²⁴ and may act as an endogenous counter-regulator of ischemia and inflammation in ischemic conditions.²⁰ The mechanism by which CGRP inhibited the flap neutrophil accumulation was not addressed in this study. However, in the rat knee joint, CGRP is effective in inhibiting histamine release from mast cells.¹⁹ Mast cells express CGRP receptors and release various cytokines and chemotactic factors.²⁵ However, it is possible that the CGRP-induced decrease of flap neutrophil accumulation is dependent on the inhibition of the release of mast cell chemotactants. In addition to 'stabilization' of mast cells, CGRP inhibits the entirely leukocyte-dependent plasma extravasation evoked by the specific leukocyte chemotactic leukotriene B₄.¹⁷ In this context, one additional possibility worth further investigation is that CGRP may suppress the expression of leukocyte adhesion molecules such as the endothelial selectins and/or leukocytic integrins. Additional documented actions by CGRP that may contribute to the beneficial effects of this neuropeptide on skin-flap survival include promotion of endothelial cell growth and inhibition of cell types such as lymphocytes, macrophages and epidermal Langerhan cells.²⁵

These results support the conclusion that intravenous administration of low doses of CGRP significantly improves the survival of surgical flaps in the rat, and indicate that the beneficial effects of CGRP may be related to a reduction in neutrophil accumulation in the flap following surgery.

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