Local Injections of Corticotropin Releasing Factor Reduce Doxorubicin-Induced Acute Inflammation in the Eyelid

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Purpose. Doxorubicin chemomyectomy is an effective alternative treatment option for patients with blepharospasm and hemifacial spasm. One side effect of the use of doxorubicin in localized injections is the development of acute inflammation and skin injury at the injection site. Corticotropin releasing factor (CRF) was reported to reduce inflammation after acute inflammatory injuries due to other causes and at other sites. This study was performed to assess the potential of CRF to prevent the development of skin injury and eyelid soreness after local doxorubicin injection.

Methods. Rabbits received lower eyelid injections of either 75 or 150 μg CRF followed by injection of either 0.5, 1, or 2 mg doxorubicin or doxorubicin alone. Eyelids were assessed for changes in acute inflammation by immunohistochemical localization of macrophages and monocytes using anti-CD11, an antibody specific for these cell types. Short-term alterations in vascular permeability were assessed using an Evans blue assay. Additional eyelids were followed daily for changes in the skin over the injection site to determine day of onset of skin injury and the total duration of skin injury. After 1 month, the eyelids were processed histologically for morphometric analysis of muscle fiber loss. Monkey eyelids also were examined for the effect of CRF and doxorubicin injections.

Results. Doxorubicin alone produced an acute inflammatory reaction in the treated eyelids, with a large influx of macrophages and monocytes throughout the connective tissue at 1 and 2 days. Corticotropin releasing factor pretreatment significantly reduced this influx of inflammatory cells into the connective tissue. Doxorubicin produced a large increase in vascular permeability in the treated eyelids, with resultant edema. Corticotropin releasing factor did not alter this change in vascular permeability, indicating that CRF appears to have a specific effect on migration of inflammatory cells rather than just a generalized effect on vascular permeability. Corticotropin releasing factor and doxorubicin cotreatments delayed the onset of skin injury and decreased the total duration of injury to the skin compared to doxorubicin alone. The effectiveness of doxorubicin chemomyectomy was maintained; muscle loss was significant at all doses of CRF combined with doxorubicin.

Conclusions. Corticotropin releasing factor dramatically decreased the acute inflammatory reaction that results in the eyelid from local doxorubicin injections. Not only did CRF reduce the acute influx of monocytes and macrophages, but it protected the skin overlying the injection site, substantially reducing the extent of skin injury. The efficacy of doxorubicin-induced muscle toxicity was maintained. A treatment protocol that combines myotoxicity with antiinflammatory activity in the treated eyelids may lead to a more effective patient treatment by increasing patient acceptance. The potential should be explored that CRF may be of clinical use in limiting tissue injury when administered immediately after extravasation during cancer chemotherapy. Invest Ophthalmol Vis Sci. 1997;38:834–841.

An effective method for the permanent nonsurgical removal of the orbicularis oculi muscle from the eyelids as a treatment for blepharospasm and hemifacial spasm is doxorubicin chemomyectomy.1–5 Doxorubicin is a potent myotoxin, and localized injection results in permanent loss of the muscle at the injection site.2,4 Although some patients with blepharospasm and hemifacial spasm have been treated successfully...
with a single injection of doxorubicin, most patients require several injections spaced over a period of months for effective reduction in muscle spasms. A major concern of the patients, however, is the acute inflammatory reaction at the injection site. The eyelid becomes swollen, red, and sensitive to the touch. After multiple injections, injury to the skin over the injection site can occur. Although the skin heals completely, with little to no scarring, the development of local inflammation, eyelid tenderness, and skin ulcers is a major concern of patients in the current doxorubicin chemomyectomy protocol. Patients would prefer a treatment that could be accomplished in a single injection, which requires that a higher dose of doxorubicin is given in a manner that does not injure the overlying skin.

The acute response of inflammatory cells to muscle injury is a complex, poorly understood phenomenon. A number of steps have been described in the process of muscle inflammation and injury. In most muscle injuries, there is an early, rapid influx of neutrophils that release cytokines into the tissue. This is followed by two separate invasions of macrophages, a first wave that removes the necrotic myofibers and a second wave that results in vasodilation and increased vascular permeability with resultant tissue edema. It is possible to monitor both these types of reactions to injury in acute inflammatory reactions by assaying for these changes in the injured muscle tissue and as well as to monitor alterations in the inflammatory reaction as a result of exposure to antiinflammatory mediators.

Corticotropin releasing factor (CRF) is a peptide of 41 amino acids that normally is produced in the hypothalamus. Local injection of CRF has reduced inflammation or vascular leakage or both after a variety of inflammation-generating injuries. These include thermal injuries, neurogenic inflammation, physical injury, drug-related injuries, and even autoimmune conditions, such as inflammatory arthritis. The potent antiinflammatory effects of CRF appear to be independent of systemic steroid release or hypotensive effects. It appears to act as an antagonist to a variety of inflammatory mediators, including histamine and substance P. Corticotropin releasing factor treatment was shown to have a specific antiinflammatory effect on skeletal muscle and skin. Localized applications of CRF prevented histamine-induced mucosal edema. Thus, CRF was a good candidate to test in the attempt to ameliorate doxorubicin-induced inflammation, swelling, and skin injury.

The mechanisms that result in acute inflammation in the eyelid after doxorubicin injection are not well understood. Doxorubicin toxicity has been postulated to result from its local production of free-oxygen radicals and the subsequent disturbances to calcium transport mechanisms. The time course of skeletal muscle necrosis in the eyelids is extremely rapid, with muscle injury present within 5 minutes of doxorubicin injection. The necrosis and removal of injured muscle continues actively for more than 1 week after doxorubicin treatment. Because of both the acute and chronic changes that occur after doxorubicin injury and the resultant muscle necrosis, CRF was chosen as a potential modulator of doxorubicin-induced injury caused by its wide array of local antiinflammatory effects in a variety of inflammatory and injury conditions. Corticotropin releasing factor was injected before doxorubicin into rabbit and monkey eyelids to determine if it could decrease the acute inflammation caused by doxorubicin treatment and protect the eyelid skin from injury. The effects of CRF and doxorubicin treatment were monitored both acutely and chronically in the treated eyelids. Because the ability of doxorubicin to kill muscle has to be maintained for its clinical effectiveness in the treatment of patients with localized muscle spasms, the effect of CRF on doxorubicin-induced muscle cell death also was determined.

**MATERIALS AND METHODS**

New Zealand White rabbits were obtained from Birchwood Valley Farm and housed with Research Animal Resources at the University of Minnesota. All animal research conformed to the guidelines from the National Institutes of Health on the use of animals in research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rabbits were anesthetized before all eyelid injections with an intramuscular injection of ketamine-xylazine, 1:1 (10 mg/kg:2 mg/kg dose, respectively). The lower eyelids of rabbits were injected with either 75 or 150 µg CRF (Peninsula Labs, Belmont, CA) in 0.4 ml sterile isotonic saline 20 minutes before the injection of either 0.5, 1, or 2 mg doxorubicin. The concentration of the doxorubicin solution was varied to result in a constant injection volume of 0.5 ml, because injection volume has an effect on the spread of drug in the eyelid. At least four eyelids were examined for each experimental parameter.

One set of rabbits was killed at 1, 2, or 4 days after CRF and doxorubicin injections and processed for the immunohistochemical localization of CD11b-positive neutrophils, macrophages, and monocytes. Briefly, the eyelids were removed from the euthanized rabbits, frozen, and sectioned on a cryostat at 12 µm. The tissue sections were fixed for 10 minutes in 10% formaldehyde solution and quenched in hydrogen peroxide to remove endogenous peroxidase. After a phosphate-buffered saline rinse, the sections were treated with blocking serum and incubated with an antibody to CD11b at a dilution of 1:40 (Accurate Chemical, Westbury, NY). The tissue was incubated using the
peroxidase ABC Vectastain Elite kit (Vector Labs, Burlingame, CA). The peroxidase labeling was visualized by incubation with diaminobenzidine and heavy metals.

A second set of rabbits was killed 24 hours after CRF and 1 mg doxorubicin injections into their lower eyelids. The eyelids were assessed for changes in vascular permeability using the Evans blue assay.18,19 The animals were anesthetized deeply and injected through the ear vein with a solution of Evans blue (50 mg/kg). After a systemic spread period of 10 minutes, the animals were perfused through the heart with a constant volume of a 1:1 phosphate-buffered saline–sucrose solution. Tissue samples were removed from each eyelid and cut to form 4 by 4-mm squares, taking care not to spread any extraneous Evans blue on the tissue samples. Each eyelid sample was placed in a test tube containing formamide and incubated in a 37°C water bath overnight. After 24 hours, the formamide solutions were assayed at 620 nm on a visible light spectrophotometer. Corticotropin releasing factor- and doxorubicin-treated eyelids were compared to eyelids treated with doxorubicin alone, CRF alone, saline alone, or no treatment. A minimum of four eyelid samples were processed for each dose examined.

The lower eyelids of a third set of rabbits were injected with CRF and doxorubicin as described in the preceding paragraphs and observed daily for 1 month after treatment. The day of onset of eyelid injury and the total duration of the skin changes were noted. After 1 month, the rabbits were euthanized and the eyelids removed for histologic examination. The tissue was frozen, sectioned on a cryostat at 12 μm, and stained histochemically for myosin ATPase. Both fast and slow fibers were counted on each section. The sections were analyzed morphometrically for changes in muscle fiber number. The CRF and doxorubicin cotreated eyelids at each of the doses were compared to eyelids treated with doxorubicin only and with control untreated eyelids. A minimum of four eyelids were prepared at each of the doses examined.

In addition, the upper and lower eyelids of four cynomolgous monkeys were injected with 75 or 150 μg CRF before the injection of either 1 or 2 mg doxorubicin. Two eyelids would be injected in a given monkey, and any skin injury or signs of inflammation were allowed to heal before the injection of the remaining two eyelids. The effect of doxorubicin alone on monkey eyelid skin and muscle loss was obtained from a previous study.19 The nonhuman primate represents the final test of safety and efficacy before proposing injections in patients. The eyelids were examined daily for the onset and duration of epithelial changes in the eyelid skin. After 1 month, these eyelids were processed for histologic and morphometric assessment of muscle fiber loss in the manner described for the rabbit lid specimens.

All results were analyzed for statistical significance using an unpaired, two-tailed t-test. An F-test indicated that the variances of the control and experimental groups were not significantly different. The statistical tests were carried out using the Instat biostatistics software (Graphpad, San Diego, CA).

RESULTS

As occurs in patients, doxorubicin injections resulted in sore eyelids that were sensitive to the touch for several weeks. In all CRF- and doxorubicin-cotreated rabbit eyelids, there was a striking decrease in the sensitivity of these eyelids to touch. In the daily examination period of the cotreated rabbits, their eyelids could be touched with no reaction of discomfort. This difference was discernible immediately and was maintained throughout the first few weeks.

One measure of acute inflammation is the influx of macrophages and neutrophils into the injured tissue. These cells can be visualized using a cell-specific antibody, CD11. At both 1 and 2 days after injection of doxorubicin only, there was a large influx of CD11-positive cells in the connective tissue of the treated eyelids (Fig. 1A). After CRF and doxorubicin cotreatments, although this was not quantified, there was a clear reduction in the number of CD11-positive cells (Fig. 1B) compared to doxorubicin alone. Normal eyelid and orbicularis oculi muscle have few CD11-positive cells within them (Fig. 1C). The CRF pretreatment resulted in a marked decrease in the intensity of the first phase of the inflammatory response to doxorubicin within the connective tissue. There did not appear to be a substantial effect on the second phase of CD11-positive cell influx, when the CD11-positive cells surround the necrotic myofibers to remove them. Many CD11-positive cells could be seen surrounding the dying myofibers in the dual-treated eyelids (Fig. 2). In the preseptal and orbital regions, the same reduction in CD11-positive cell influx could be seen after CRF and doxorubicin treatments compared to doxorubicin alone (Fig. 3). By reducing the connective tissue influx of CD11-positive cells, while not eliminating the cells that remove necrotic muscle fibers, muscle loss would be maintained.

Injury and inflammation also result in increased vascular permeability and edema. The effect of CRF on vascular permeability was examined in rabbits 1 day after treatment using the Evans blue assay. Doxorubicin substantially increases the vascular permeability in the treated eyelids compared to uninjected control,19 saline-injected, or CRF-injected control eyelids (Fig. 4). The injection of CRF before the injection of doxorubicin did not diminish the doxorubicin-induced increase in vascular permeability. At the higher
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FIGURE 1. Cross-sections through rabbit eyelids stained for the presence of CD11-positive cells (black) 1 day after injections with (A) doxorubicin only, (B) corticotropin releasing factor (CRF) and doxorubicin, and (C) uninjected control. Doxorubicin alone results in influx of CD11 cells (A), whereas CRF reduces their presence (B). Bar = 100 μm.

dose of CRF combined with doxorubicin, there was an apparent increase in vascular permeability compared to doxorubicin alone. This difference was not significant. Thus, although CRF was able to decrease the influx of macrophages and monocytes, it did not have an effect on the development of edema in the treated eyelids 1 day after doxorubicin injection.

As has been shown in previous studies, all eyelids that were injected with doxorubicin alone develop skin injury (Fig. 5). This usually appears within the first week. These sores heal completely, but this process can take longer than 30 days. With CRF pretreatment, there was a delay in the onset of skin injury (Fig. 5). Most significantly, there was a dramatic decrease in the total duration of skin injury in the cotreated eyelids (Fig. 5). This decrease in the duration of skin injury was significant in all CRF and doxorubicin cotreated eyelids at all doses of doxorubicin except 150 μg CRF and 2 mg doxorubicin. A similar picture was seen after CRF and doxorubicin injections into monkey eyelid (Fig. 6). Onset of epithelial changes was delayed, and the duration was decreased significantly at all the doses examined.

Although it is important to decrease tissue inflammation in the doxorubicin-treated eyelids, it also is important to maintain the muscle loss, which, in turn, is responsible for its clinical effectiveness in the treatment of muscle spasm diseases. Morphometric analyses of the eyelids indicated that although CRF pretreatment significantly protected the skin from injury, the level of doxorubicin-induced muscle loss was maintained in the CRF and doxorubicin-treated eyelids in both rabbit (Figs. 7, 8) and monkey eyelids (Fig. 9). At doxorubicin doses of either 1 or 0.5 mg, CRF pretreatment increased significantly muscle loss over doxorubicin alone. Thus, CRF pretreatment maintained or improved the myotoxic effectiveness of doxorubicin injections into the eyelids.

DISCUSSION

The injection of CRF into the eyelids of rabbits before doxorubicin injection reduced significantly the acute
inflammatory reaction in the treated eyelids as assessed by immunohistochemical localization of macrophages and monocytes in the connective tissue of these eyelids. The tissue noticeably was less red and less sensitive to the touch than were eyelids treated with doxorubicin alone. Corticotropin releasing factor delayed slightly the onset of epithelial changes overlying the injection site and decreased significantly the duration of epithelial changes to the eyelid skin. Concomitant with the significant decrease in inflammation and skin injury, the efficacy of doxorubicin-induced muscle toxicity and loss was maintained. Muscle loss was equal to or greater than that seen with doxorubicin alone.

The initiation and control of inflammation is a complex, multistep phenomenon. Whereas a variety of drugs and injuries can produce acute inflammation, the subsequent processes that occur within the injured tissue seem to be fairly similar. There is an immediate influx of neutrophils, which release cytokines into the tissue. This is followed by sequential invasions of macrophages into the connective tissue and ultimately surrounding the necrotic muscle fibers. These macrophages release additional cytokines, which further stimulate the inflammatory process. The exact mechanisms by which corticotropin releasing factor inhibits inflammation are not fully understood, but it is likely that it acts through the release of anti-inflammatory cytokines and the suppression of pro-inflammatory cytokines. Further studies are needed to elucidate the specific mechanisms involved.

FIGURE 3. Cross-sections through the preseptal and orbital regions of rabbit eyelids 1 day after injection of (A) 1 mg doxorubicin, (B) 150 µg corticotropin releasing factor (CRF) and 1 mg doxorubicin. A higher power view through the preseptal region of rabbit eyelids 1 day after injection of (C) 1 mg doxorubicin and (D) 150 µg CRF and 1 mg doxorubicin. Note that there are fewer CD11-positive cells in the CRF and doxorubicin cotreated eyelids (B,D) than in the doxorubicin only treated lids (A,C). Muscle fibers are indicated by arrows. CD11 cells are black. Bar = 100 µm.

FIGURE 4. The effect of corticotropin releasing factor on doxorubicin induced increases in vascular permeability as visualized with the Evans blue assay. Bars indicate standard errors.

FIGURE 5. The effect of corticotropin releasing factor on the time of onset and the duration of skin injury in rabbit eyelid caused by doxorubicin chemomyectomy. Bars indicate standard errors.
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FIGURE 6. The effect of corticotropin releasing factor on the time of onset and the duration of skin injury in monkey eyelid caused by doxorubicin chemomyectomy. Bars indicate standard errors. d X n only dose was 2 mg.

Each of the different reactions to injury probably is the result of different signals released by the injured tissue.

The antiinflammatory effects of CRF are well documented. Corticotropin releasing factor has been shown to exert its effects after a variety of injuries, including thermal, chemical, physical, and even after direct neurogenic stimulation. A strain of rats with increased susceptibility to inflammatory disease was shown to be deficient in CRF, reinforcing the normal role of CRF in modulating inflammatory disease. The most characteristic response to the systemic administration of CRF is the resultant decrease in plasma extravasation and vascular permeability elicited by the various types of injury. Whereas the usual method of CRF administration in these studies was systemic, local injections of CRF were equally efficacious in decreasing vascular permeability changes. Corticotropin releasing factor also has antinociceptive properties. Corticotropin releasing factor exerts an effect on monocytes and macrophages but not lymphocytes. In the current injury model, CRF did not prevent increased vascular permeability that is caused by doxorubicin treatment within the eyelid during the

FIGURE 7. Cross-sections through rabbit eyelids stained with the myosin ATPase histochemical procedure to visualize myofibers. The eyelids are (A) an un.injected control or were treated with (B) 150 μg corticotropin releasing factor (CRF) and 0.5 mg doxorubicin or (C) 150 μg CRF and 2 mg doxorubicin. Orbicularis oculi muscle fibers are indicated by arrows. Bar = 100 μm.

FIGURE 8. Morphometric analysis of muscle fiber number in rabbit eyelids treated with doxorubicin or doxorubicin and corticotropin releasing factor (CRF) compared to control untreated eyelids. All are significantly different from the control eyelids. There is no significant difference in the muscle fiber numbers between the eyelids treated with doxorubicin only compared with those lids treated with CRF and doxorubicin. Bars indicate standard errors.
FIGURE 9. Morphometric analysis of muscle fiber number in monkey eyelids treated with doxorubicin or doxorubicin and corticotropin releasing factor (CRF) compared to control untreated eyelids. All are significantly different from the control eyelids. There is no significant difference in the muscle fiber numbers between the eyelids treated with 2 mg doxorubicin only compared with those lids treated with CRF and doxorubicin. Bars indicate standard errors.

The complexity of tissue reactions to injury makes it difficult to find a good antiinflammatory mediator. As in the current study, CRF was able to reduce the acute influx of macrophages and monocytes and protect the skin from injury, but did not alter the edema caused by the doxorubicin. Luckily, the CRF did not alter the effectiveness of doxorubicin in killing muscle. Another antiinflammatory mediator, cyclosporin, reduced both the skin injury and vascular permeability changes caused by doxorubicin alone, but did not alter tissue sensitivity to touch. Unfortunately, cyclosporin also protected the muscle from the desired doxorubicin myotoxicity. These drug studies indicate the difficulty in trying to ameliorate localized tissue inflammation. The complexity and multifaceted nature of each tissue's response to a specific inflammatory mediator makes protection from injury difficult.

The effect of doxorubicin on epithelium has been described during its use in intravenous chemotherapy for cancer treatment. Extravasation can result in large injuries to the skin over the site of exposure. Increased spread of the doxorubicin, by coinjection of the drug with collagenase or bupivacaine and hyaluronidase, increased skin injury, presumably by increasing its ability to reach and therefore injure the skin. When doxorubicin injections followed a cyclosporin pretreatment, skin injury was reduced greatly and, in some cases, prevented completely. CRF has a known antiinflammatory effect on skin and the subcutaneous injection of CRF in the eyelid may have facilitated the protection of the skin from doxorubicin injury.

Although CRF had a prominent effect on reducing the influx of monocytes and macrophages in the connective tissue after the doxorubicin injections, it did not seem to alter the influx of macrophages around the necrotic muscle fibers (Fig. 2). This may help explain why the muscle injury was not diminished by the CRF treatment of the eyelid. This is in marked contrast to the effects of cyclosporin pretreatment before doxorubicin-induced injury where the muscle, unfortunately, was protected from the myotoxic effects of doxorubicin. This resulted in a diminished effectiveness in killing the muscle for the purpose of preventing muscle spasms. It may be that the tissue half-life of each of these drugs differed after direct eyelid injection. Cyclosporin is lipophilic and may remain at the injection site for a longer period than would CRF. This may, in part, account for CRF effects in the first phase of inflammation only. The different mechanism of action of these two drugs must play a role in their differential effects on the injured muscle fibers.

The decrease in inflammation and soreness at the injection site and the simultaneous decrease in skin injury represent a major improvement in the doxorubicin chemomyectomy protocol as a treatment method for blepharospasm and hemifacial spasm. Local skin inflammation and sensitivity to touch are the major concerns of patients receiving doxorubicin chemomyectomy for treatment of their muscle spasm disease. This improvement should increase patient acceptance of doxorubicin chemomyectomy as a treatment alternative, adding to the safety of the treatment while maintaining the effectiveness of doxorubicin in producing permanent muscle loss. Immediate injection of CRF after an extravasation during cancer chemotherapy may help prevent the skin injury that occurs after this inadvertent exposure.

Key Words
blepharospasm, corticotropin releasing factor, doxorubicin, hemifacial spasm, inflammation, myotoxicity, orbicularis oculi

References
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