Comparison between the Effects of Dexamethasone and Indomethacin on Bone Wound Healing

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Abstract—The area of wound holes made in the parietal bone of 4-week-old rats was measured at 1, 2 and 4 weeks after the operation using Alizarin red S staining without the use of histological sections. Dexamethasone and indomethacin were administered s.c. after the operation every day except Sunday. The inhibitory action of dexamethasone on bone wound healing was stronger than that of indomethacin, as was its inhibitory action on skin wound healing. Inhibition of the age-related decrease in radiolucency of femurs and some changes in serum calcium level were also observed by dexamethasone administration, but not by indomethacin. These results suggest that dexamethasone inhibits bone wound healing through its effects on calcium metabolism in addition to its general inhibitory action on wound healing which is shared with indomethacin. Dexamethasone also strongly inhibited the decrease in Alcian blue stainability which occurs with an increase in age, both in the normal portion and in the wound hole portion of the calvaria, whereas indomethacin showed a weak inhibitory effect only in the wound hole portion. This result suggests that one of the mechanisms by which dexamethasone inhibits bone wound healing may be based on the retardation of mineralization caused by the inhibition of removal of acid mucopolysaccharide.

Several anti-inflammatory drugs are widely used for the treatment of bone fractures and the wound caused by tooth extraction. However, the effect of these drugs on the wound healing process in bone is not well understood yet.

Many reports (1–4) indicate that steroidal anti-inflammatory drugs inhibit bone wound healing. In the case of non-steroidal anti-inflammatory drugs, indomethacin is reported to retard fracture healing in man (5) and rats (6) and tooth extraction wound healing in rats (7).

Most of these reports were based on histological observations, though some involved biochemical or radioisotope studies. Few morphometric analyses have been carried out. There is also little information on the difference between the mode of inhibitory action of steroidal and non-steroidal anti-inflammatory drugs.

In the present study, the differences between the effects of dexamethasone as a representative steroidal anti-inflammatory drug and indomethacin as a nonsteroidal anti-inflammatory drug on bone wound healing were examined morphometrically.

Materials and Methods

Male Wistar strain rats, 4 weeks of age, were used in the present study. Under pentobarbital anesthesia, the skin of the parietal region was incised and holes were drilled in both parietal bones of the calvaria using a round bur with a diameter of 0.8 mm (No. 009, Komet, West Germany), as shown by the arrows in Fig. 1.

A mixture of penicillin (5000 U/ml) and streptomycin (10 mg/ml) was administered both systemically (0.2 ml/100 g, i.m.) and locally as a prophylaxis against infection.
The animals received subcutaneously 0.5 or 2.0 mg/kg of dexamethasone (Sigma, St. Louis, U.S.A.) or indomethacin (Sigma) suspended in 0.5% carboxymethylcellulose solution containing 5% ethanol every day except Sunday after the operation for 1, 2 or 4 weeks. The rats administered 0.5% carboxymethylcellulose containing 5% ethanol served as a control. Some rats died in the middle of the experiment following administration of 2 mg/kg of dexamethasone, so the longest possible period of administration was 16 days for that dosage.

Rats were killed at 1, 2 or 4 weeks after the operation, and the calvaria containing the wound holes was isolated. Blood was collected from the heart for the analysis of the serum calcium level, and femurs were also removed for X-ray observation.

The calvaria was fixed with 95% ethanol for 10 days. After the fixation, both the right and left portions of the calvaria was separated sagittally, and the right portion containing the wound hole was first immersed in 2% KOH for 3 to 5 days and then stained for 12 to 24 hr with 0.01% alizarin red S dissolved in 1% KOH for the detection of calcified areas.

After staining, they were placed in a 10% glycerin solution containing 1% KOH to adjust the staining intensity to a suitable level, then passed through a graded series of glycerin solutions, and finally placed in 100% glycerin for microscopic observation and photography. The other side of the calvaria was stained with 1% Alcian blue adjusted to pH 2.6 with 3% acetic acid. The pieces of the bone were washed with purified water 3 times, 10 min each time, both before and after the staining. Then they were passed through a graded series of ethanol solutions and placed in 95% ethanol for microscopic observation and photography. After determination of the intensity of Alcian blue staining, the specimens were stained again, this time with alizarin red S as mentioned above.

Pieces of the bone stained with alizarin red S or doubly-stained with alizarin red S and Alcian blue were photographed using a microscope with a camera attachment, and the area of the hole on the photograph, (i.e., the unstained portion by alizarin red S staining), which decreases in inverse proportion to the area of newly formed bone, was measured with a Summagraphics Digitizer-ID DATA TABLET/DIGITIZER (Summagraphics Corp., Connecticut, U.S.A.) connected to a Canon Desktop Computer-Canon BX-1 (Canon Co., Tokyo, Japan).

The intensity of Alcian blue staining for acid mucopolysaccharide was classified into 5 degrees (+4: strong, +3: moderate, +2: weak, +1: slight, 0: background only) and was determined for both the normal portion and wound hole portion of the bone. From among the total number of preparations, five preparations, each representing one of these five degrees, were selected and used as standards for comparison with the other preparations.

Soft X-ray photographs of the femurs were taken with a soft X-ray apparatus (Softex type CMB2, Softex Co. Ltd., Tokyo, Japan) at 17 kvp, 2 mA, for 2 min (FFD 37 cm). The level of calcification of the diaphysis was assessed in terms of the degree of radiolucency on X-ray films.

Serum calcium levels were determined by the method of Morin (8).

A skin wound, approximately 10 mm in diameter, was made with scissors by resection of the dorsal skin of rats, and the area of the wound was measured 6 days after the operation.
Results

1. Typical morphological and histochemical changes in the parietal bone and wound hole area in control rats: The area of the wound hole in rats receiving only the drug vehicle decreased with the progression of time, as shown in Fig. 2a.

Both stainability of the wound hole portion and that of the normal portion of calvaria for Alcian blue decreased with time (Fig. 2b). The preparations stained with Alcian blue could also be used to measure the wound hole size after double staining with alizarin red S (Fig. 2c).

2. Changes in the wound hole area following drug treatment: The changes in the wound hole size in the parietal bone following administration of dexamethasone or indomethacin are shown in Fig. 3. Both control and drug-treated groups showed decreased areas of the holes as a function of time, and a significant inhibition by dexamethasone of the wound healing was observed on the 4th week (Fig. 3a).

The inhibitory effect of indomethacin on wound hole healing was not so strong as that of dexamethasone, but a significant inhibition was observed on the 4th week (Fig. 3b).

3. Changes in the intensity of Alcian blue staining: As shown in Fig. 4, the Alcian blue stainability of the wound hole portion in the control group decreased with increasing time after the wounding.

When dexamethasone or indomethacin was administered for 1 week, no clear difference in Alcian blue staining was observed among control, dexamethasone, and indomethacin groups at the wound hole portion of the parietal bone. However, both dexamethasone and indomethacin showed an inhibitory effect on the decrease in Alcian blue stainability at the 2nd and 4th weeks, with the inhibition by dexamethasone being stronger than that by indomethacin.

The Alcian blue stainability of the normal portion (non-wounded portion of calvaria) in the control group decreased faster than in the wound hole portion. An inhibitory effect of dexamethasone on the decrease in Alcian blue stainability was found at the 2nd and 4th weeks, but the effect of indomethacin was not so clear as that of dexamethasone (Fig. 5).

4. Results of X-ray observation of rat femurs: In Table 1, the degrees of radiolucency of the diaphysis of femurs from the experimental groups are arranged in descending order. As shown there, the radiolucency of the diaphysis of femurs from the control group decreased with the progression of time, while that of femurs from the dexamethasone-treated groups at the 2nd or 4th week was

![Fig. 3. Changes in the wound hole area of rat calvaria following administration of dexamethasone and indomethacin. Each point and vertical bar represents the mean±S.E. of 5 or 6 holes. *P<0.05, **P<0.001: Significant difference from the corresponding control group (Student's t-test).](image-url)
Fig. 2. Wound hole portions of rat calvaria stained with alizarin red S and/or Alcian blue (Original magnification, 10×)
Fig. 4. Effects of dexamethasone and indomethacin administration on Alcian blue staining of the wound hole portion of calvaria. C: Control, DM: Dexamethasone, IM: Indomethacin, \( \bar{I} \): Mean of the intensity of Alcian blue staining of each wound hole portion (+).

Fig. 5. Effects of dexamethasone and indomethacin administration on Alcian blue staining of the normal portion of calvaria. C: Control, DM: Dexamethasone, IM: Indomethacin, \( \bar{I} \): Mean of the intensity of Alcian blue staining of each normal portion (+).

Table 1. Results of X-ray observations of the femur of rats which had been administered dexamethasone or indomethacin (Radiolucency in descending order)

| Group          | 1 Week | 2 Weeks | 4 Weeks |
|----------------|--------|---------|---------|
| Control        |        |         |         |
| 1 Week         | DM 2.0 | DM 0.5  | DM 0.5  |
| 2 Weeks        | DM 2.0 | DM 0.5  | DM 0.5  |
| 4 Weeks        | DM 2.0 | DM 0.5  | DM 0.5  |

DM: Dexamethasone, IM: Indomethacin.
stronger than that of the control group. However, for the indomethacin-treated groups, the results were almost the same as those of the control group. This suggests that dexamethasone inhibits calcification, but indomethacin does not.

5. Results of skin wound healing: As shown in Fig. 6, both dexamethasone and indomethacin had inhibitory effects on skin wound healing, but the dexamethasone effect was greater than that of indomethacin.

6. Changes in serum calcium levels: The results of the effects of dexamethasone and indomethacin administration on serum calcium levels are shown in Fig. 7. Dexamethasone caused an increase in the serum calcium level at the 1st and 2nd weeks, but the level was somewhat decreased at the 4th week by 0.5 mg/kg of dexamethasone (Fig. 7a). Differently from dexamethasone, indomethacin had no significant effect on the serum calcium level (Fig. 7b).

Discussion

Several experimental techniques or methods, such as histological and histochemical examinations, biochemical and radiographic analyses, determination of tensile strength properties, and experiments using radioisotopes (e.g., $^{85}$Sr), have been applied to the! study of the effect of drugs on wound healing of experimental fractures of long bones or tooth extraction wounds in experimental animals.

These experimental methods or techniques are useful and can provide important information. However, the structure of the fractured bone and the tooth extraction cavities are complicated, and some of these experimental methods are also complicated. So, these experimental materials and methods are not always suitable in many animals, especially when statistical evaluation of the effect of a drug on the degree of bone regeneration after wounding is required within a short time. The parietal bones of rat calvaria are thin and board-like, having an almost uniform thickness of about 500 μm at 8 weeks of age. It was possible to make wound holes of almost uniform size in the bone and to stain preparations without making ordinary histological sections in a comparatively short time. The preparation of regenerating bone after the wounding could be observed under low power magnification (×10). The wound hole of the parietal bone is considered to be a simplified suitable model for the more complicated bone...
wounds such as those that occur as results of tooth extraction.

The mechanism of the staining reaction of alizarin red S (which has been extensively used in studies on bone growth) has recently been found by Virtanen and Isotupa (9) to involve chelate formation between the stain and calcium on the bone surface. Therefore, the part of the sample preparation which is stained red with alizarin red S in the present experiment is the calcified portion, and the degree of newly formed bone can be known by substitution of the area of the unstained remaining hole area from the original area of the wound hole.

In another of our experiments (10), the result of the morphometry of the bone wound hole area with alizarin red S staining and the result of that with radiography well paralleled one another.

The present study revealed a strong inhibitory action of dexamethasone not only on wound hole healing of parietal bones but also on skin wound healing. The normal decrease in radiolucency of femurs was also inhibited, and some changes in serum calcium level were observed following dexamethasone administration. Nordin et al. (11) noticed that the effect of corticosteroids on calcium metabolism is mainly manifested in reduced calcium absorption, which produces increased bone resorption. These results suggest that dexamethasone inhibits bone wound healing by its influence on calcium metabolism, in addition to its inhibition of the synthesis of ground substances, the latter being one of its effect on connective tissue (12, 13).

Indomethacin, on the other hand, showed weaker inhibitory effects than those of dexamethasone on bone wound healing and skin wound healing, but no effects were observed on serum calcium level or roentgenograms of the femur, suggesting inhibition at an earlier stage of the wound healing process than mineralization. This result agrees with that of Kato and Ogura (14) who showed that sodium salicylate inhibited experimental ectopic calcification and decreased the plasma calcium level, whereas indomethacin did not.

The facts that the degree of Alcian blue staining progressively decreased with time in both the wound hole portion and the normal portion of the parietal bone in control rats and the decrease of the stainability was strongly inhibited by dexamethasone administration suggest a close relationship between the decrease in acid mucopolysaccharide and mineralization of bone. Kostenszky and Olah...
(4) also noticed delayed development, higher Alcian blue positivity, and a greater chondroitin sulfate content of the callus of fractured tibiae of prednisolone-treated dogs than in that of control dogs. Their result and ours are in accord with the thesis of Jibril (15) that the removal of sialoprotein or mucopolysaccharide is important for mineralization, and the result of Alcian blue staining suggests retardation of mineralization caused by the inhibition of removal of acid mucopolysaccharide as an inhibitory mechanism of dexamethasone in bone wound healing.

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