

A Radioautographic Study of Healing Following Simple Gingivectomy. II. The Connective Tissue

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THIS PAPER is part II of a study on healing following simple gingivectomy in rhesus monkeys. The first paper¹ reported epithelial regeneration; this paper is concerned with the connective tissue healing and regeneration of the free gingiva. Tritiated thymidine radioautography was utilized, in addition to conventional histologic methods, in order to locate in a sequential order the areas of proliferative potential and the dynamics of connective tissue proliferation during healing of a gingivectomy wound.

MATERIALS AND METHODS

The experimental procedures used in this investigation were described in detail in the previous paper on epithelial regeneration.¹ As a brief summary it can be stated that simple gingivectomies involving posterior teeth were performed in three adult male rhesus monkeys at time intervals of 35, 21, 14, 9, 7, 5, 3, 2 days, and 37, 25, 13, 9, 5, and 2 hours prior to sacrifice of the animals. One hour before each animal was killed (at 10 a.m.) it received intravenously 1 μ c. per gram of body weight of tritiated thymidine (Specific activity 6.7 curies per mm). The jaws with contiguous soft tissues were fixed in 10% neutral buffered formalin, decalcified in 20% EDTA adjusted to a pH of 7.4 with NaOH, embedded in paraffin, and sectioned at 8 μ . Radioautographs were prepared by the dipping method and the underlying sections were stained with Ehrlich's acid hematoxylin. A number of sections not exposed to the radioautographic technique were stained with hematoxylin and eosin for comparative histologic study.

The administration of tritiated thymidine and subsequent preparation of radioautographs locates the cellular synthesis of DNA, since thymidine is in-

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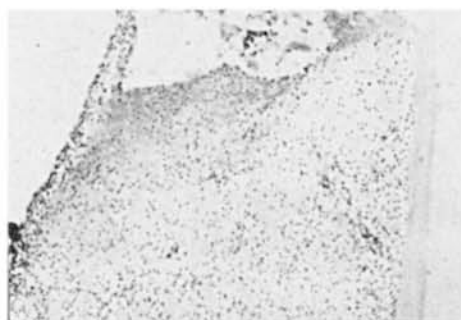


Fig. 1A. Gingivectomy wound 5 hours after surgery. Beginning establishment of "poly-band" at wound surface. (Magnification X55).

corporated into cells during the S-phase of their premitotic interphase.^{2, 3} When thymidine is tagged with tritium, the cells synthesizing DNA at the time of administration can be demonstrated in radioautographs. The incorporation of tritiated thymidine into newly forming DNA starts within one minute after the intravenous injection of the isotope and the incorporation is nearly complete within 10 minutes.⁴ Because of this rapid incorporation of tritiated thymidine into cells in the premitotic phase, and since the animals were allowed to live one hour after the administration of the thymidine there is one hour time differential between the histologic and radioautographic observations in the reported material. For instance, radioautographs depicting cells synthesizing DNA one hour following gingivectomy were superimposed on histologic slides that show the histologic changes 2 hours following the surgery, since the histologic changes continued after the injected isotope already had been deposited in the cells. Sections and radioautographs from non-operated areas in the experimental monkeys were available for control.

An approximately 3 mm wide gingivectomy wound was created buccally and lingually at each of the experimental teeth, and the interproximal papillae were removed. The alveolar crest was not exposed. No postoperative surgical dressing was used since it has been found in other studies of monkeys that such dressings in many instances interfere with the process of healing

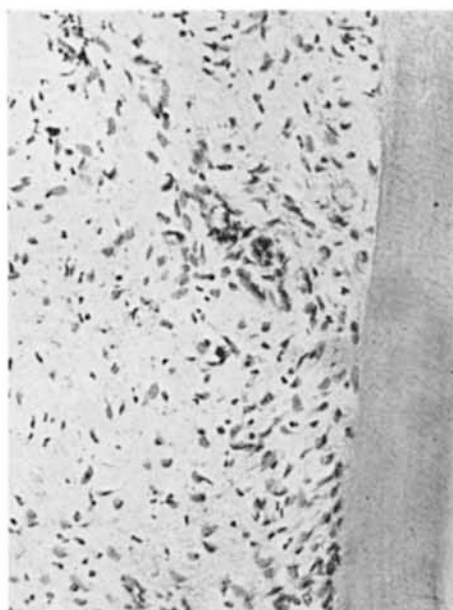


Fig. 1B. High magnification from Fig. 1A of contact between tooth and gingiva. Note loss of cementoblasts at upper $\frac{2}{3}$ of picture. (Magnification X225).

and make it impossible to standardize the experimental conditions under which healing takes place.

FINDINGS

As early as 2 hours following the surgery, the wound was covered by a blood clot and there was a marked migration of polymorphonuclear cells into the clot and to the surface area of the wound. A thin layer, approximately 0.2 mm thick immediately adjacent to the surgical incision showed widely spaced, pale staining connective tissue cells. In the 5 and 9 hours specimens it could be observed that this thin layer of degenerating cells gradually became filled with polymorphonuclear cells, (Fig. 1A), so that 13 hours after the surgery there was a band of polymorphonuclear cells covering the wound. This "poly-band" included the superficially degenerated and necrotic connective tissue resulting from the surgical procedure and also part of the surface blood clot. The cementoblasts were disrupted and absent from the cemental surface for a depth of approximately 0.2 mm from the surgical wound

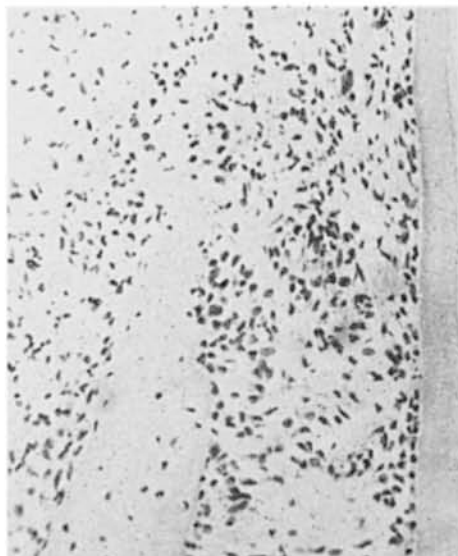


Fig. 2. Loss of osteoblasts on outer surface of alveolar crest 13 hours after gingivectomy. (Magnification X225).

surface (Fig. 1B). Under the surface zone of heavy polymorphonuclear infiltration, a few polymorphonuclear leukocytes could be found around small blood vessels, but except for at the wound surface there was a very little, if any, inflammatory reaction up till 13 hours after the gingivectomy. The radioautographs did not show any increase in number of labeled cells compared with the control material, and no labeled connective tissue cells were seen at the wound margin up to 12 hours after surgery.

In several sections there appeared to be a loss of the continuity of the osteoblastic layer at the outer aspect of the alveolar crest (Fig. 2). This was first observed 13 hours following the gingivectomy and was found in sections up to 3 days after the surgery. However, other sections from the same time intervals showed almost undisturbed osteoblasts present at the crest. It is conceivable that the pressure from the surgical procedures had a transient depressive effect upon the osteoblastic activity. It may also have been that the inflammatory response from the wound had extended to the periosteum in selected areas although this assumption could not be verified on

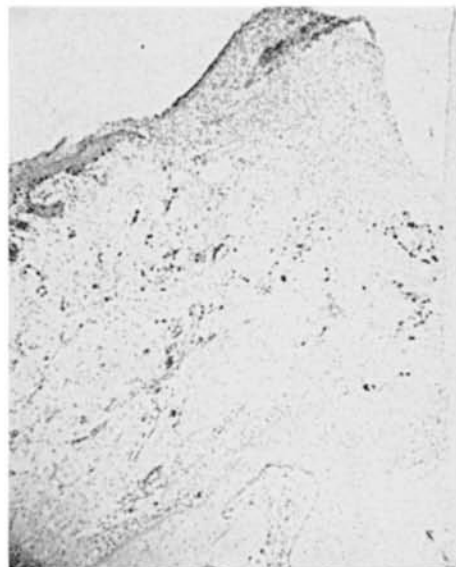


Fig. 3A. Labeled cells between wound and alveolar crest 24 hours after gingivectomy. (Magnification X55).

the basis of distribution of inflammatory cells.

The first increase in number of labeled connective tissue cells in the wound area was noted in the 24 hour radioautographs which were superimposed on histologic sections showing healing 25 hours following surgery (Fig. 3A). Most of the labeled cells were found about 0.3 to 0.5 mm below the polymorphonuclear band covering of the wound and clearly above the periosteum of the alveolar crest. Most of the labeled cells appeared to be angioblasts, either within the wall or in the immediate vicinity of the walls of small blood vessels (Fig. 3B). The labeling of cells around blood vessels also extended into the superior portion of the periodontal membrane and into the approximating marrow spaces. A few spindle shaped perivascular cells and occasional small round cells resembling lymphocytes were labeled. In these short term specimens most of the labeled connective tissue cells were adjacent to inflammatory cells, (mainly polymorphonuclear cells).

Two days following the gingivectomy there was a further increase in the number

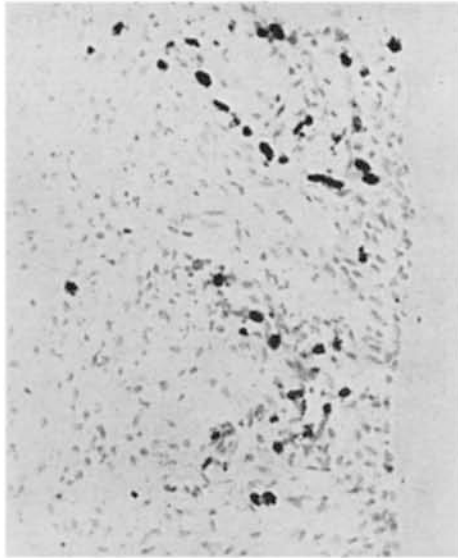


Fig. 3B. High magnification from Fig. 3A. showing labeled cells, most of them in vessel walls. (Magnification X210).

of labeled endothelial and perivascular cells (Fig. 4A). The labeled cells were still about 0.3 to 0.5 mm under the polymorphonuclear band covering the surface of the wound. Inflammatory cells, mainly lymphocytes were widely distributed in the perivascular tissues, both in the supracrestal areas and extending into the periodontal membrane. Under the "poly-band" there were rarely any polymorphonuclear cells in the tissues. A few labeled cementoblasts were seen in the areas where the inflammatory reaction extended towards the cementum (Fig. 4B). There were no labeled osteoblasts at the alveolar crest.

Three days following the gingivectomy, labeled endothelial cells and fibroblasts also were concentrated mainly in the zone 0.3 to 0.5 mm under the surface "poly-band" and clearly above the periosteum of the alveolar crest (Figs. 5A and 5B); however, towards the original wound margin where the connective tissue now was covered by epithelium, labeled cells were distributed up to the basal cell layer of the epithelium. In the locations of labeled cells there were a few scattered polymorphonuclear cells and some lymphocytes in a perivascular

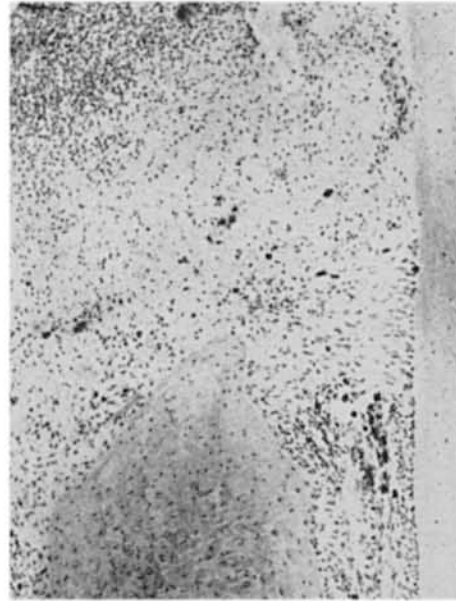


Fig. 4A. Labeled cells between "poly-band" and alveolar crest. Labeled cells also associated with blood vessels in periodontal membrane. Note labeled cementoblast. Osteoblasts absent on outer surface of alveolar crest. Two days after surgery. (Magnification X105).

arrangement. A few cells that appeared to be cementoblasts were labeled.

Five days after the gingivectomy the surface of the wound was partially covered by a thin layer of epithelium which had separated the superficial layer of polymorphonuclear cells and debris from the underlying connective tissue (Figs. 6A and 6B). The inflammation was more diffusely distributed in the supracrestal tissues than in the 3 day specimens. However, in areas where the epithelium did not cover the wound, the labeled cells did not extend to the surface (Fig. 6C). A few polymorphonuclear leukocytes appeared near the newly formed epithelium and at the uncovered border of the wound toward the surface of the tooth. From the second day onward the inflammatory cells were predominately lymphocytes except in the immediate vicinity of the wound surface. Labeled connective tissue cells were found immediately under the newly formed epithelial covering of the wound. The connective tissue close to the new epithelial coverage was embryonal in character, and had many thin



Fig. 4B. Another radioautograph from same specimen as Fig. 4A. Zone of maximum labeling between wound surface and alveolar crest. Most labeling associated with vessels and inflammation. Labeled cementoblasts 2 days postoperatively. (Magnification X118).

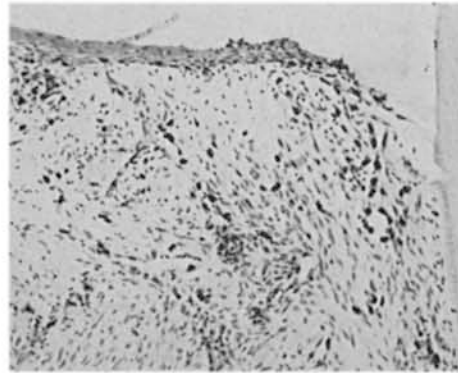


Fig. 6A. The new epithelium covering the wound. Labeling of connective tissue cells up to basement membrane of epithelium. Notch in tooth from gingivectomy knife. Five days postoperatively. (Magnification X135).

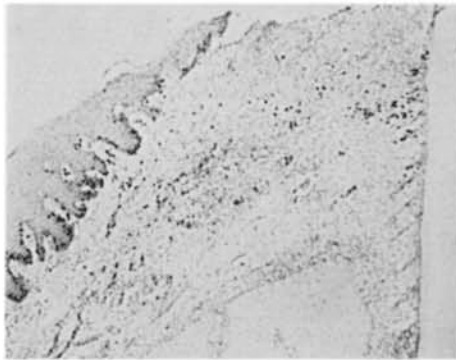


Fig. 5A. Concentration of labeled cells between wound and alveolar crest. Marked labeling of basal cells of epithelium. Three days postoperatively. (Magnification X55).

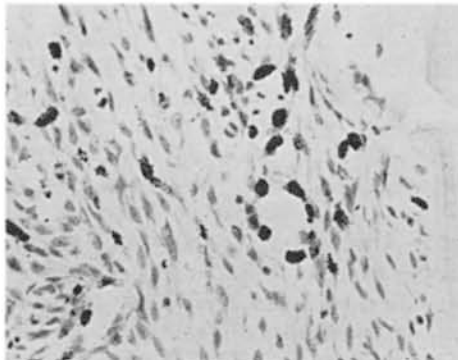


Fig. 6B. High magnification from Fig. 6A. Note labeled angioblasts. (Magnification X285).

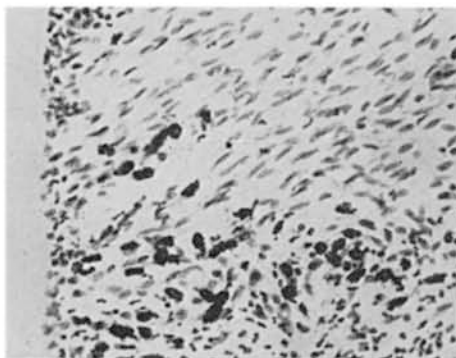


Fig. 5B. High magnification from Fig. 5A. Labeling of angioblasts, fibroblasts and small round cells resembling lymphocytes. (Magnification X285).

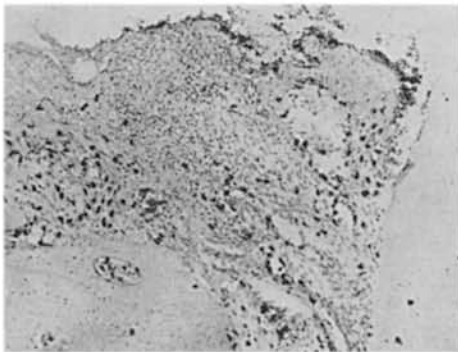


Fig. 6C. Area not completely covered by epithelium in 5 day specimen. Labeled cells spread in supracrestal tissues and into periodontal membrane. Also labeling in marrow spaces. (Magnification X103).

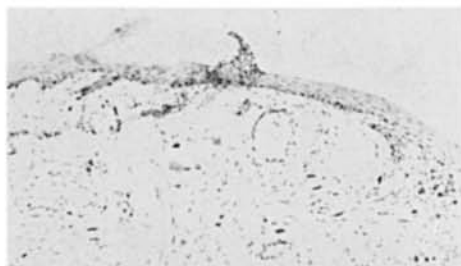


Fig. 6D. Vascular and embryonic character of granulation tissue under regenerated epithelium. Labeling both in connective tissue and epithelium. Five days postoperatively. (Magnification X105).

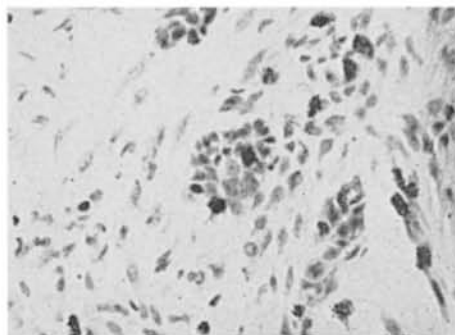


Fig. 6E. Labeled Malassez's epithelial rests in 5 day specimen. (Magnification X380).

walled vessels and plump widely spaced fibroblasts in a delicate connective tissue stroma (Fig. 6D). A few labeled osteoblasts were seen at the alveolar crest. Labeling was also seen in Malassez's epithelial rests of the supracrestal connective tissue (Fig. 6E).

At 7 days a new free gingiva had formed as a result of connective tissue proliferation. The outer gingival surface was completely epithelialized and there was beginning epithelialization of the sulcus area (Fig. 7). The connective tissues of the newly formed marginal gingiva was highly vascular and contained many large fibroblasts and numerous lymphocytes, diffusely distributed in the crevicular part of the free gingiva. Most of the labeled cells still were endothelial cells, but a number of labeled fibroblasts were also observed. The total number of connective tissue cells synthesizing DNA decreased between the 5th and 7th day, and the connective tissue making up the newly formed free gingiva appeared more mature in the 7 than in the 5 day specimen. Several collagen bundles were present in the connective tissue above the level of the original incision in the 7 day specimen. At the alveolar crest there were several unlabeled osteoclasts and some labeled osteoblasts present.

Nine days following the gingivectomy there was more osteoclastic activity at the alveolar crest than at any other time. In addition to the unlabeled osteoclasts present there were several labeled osteoblasts present on the surface of the alveolar crest

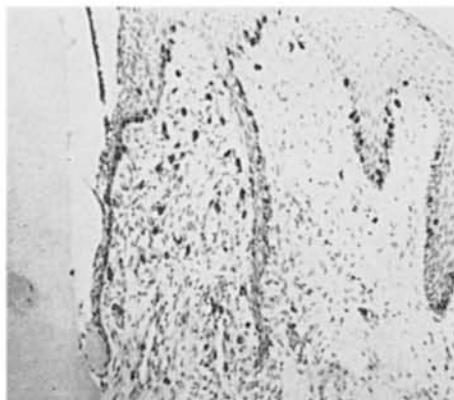


Fig. 7. Seven days after gingivectomy. Intensive labeling of granulation tissue bordering the new gingival sulcus. (Magnification X87).

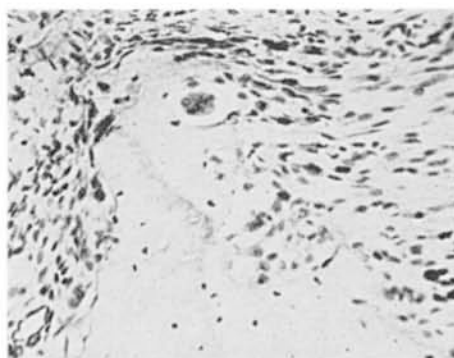


Fig. 8. Osteoclastic and osteoblastic activity at the alveolar crest in the 9 day specimen. (Magnification X250).

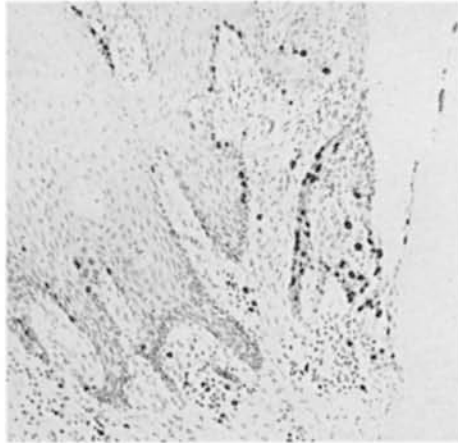


Fig. 9A. Labeled cells and chronic inflammation to the bottom of the new epithelial attachment. Twenty-one days after surgery. (Magnification X78).

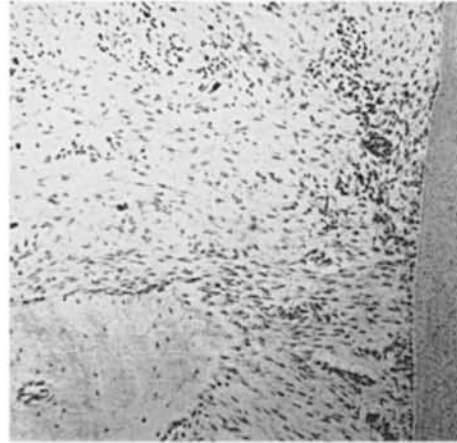


Fig. 9B. From same specimen as Fig. 8A. Irregular arrangement of cementoblast at top $\frac{1}{2}$ of picture. No labeled cells at alveolar crest. (Magnification X87).

(Fig. 8). Although the gingival sulcus was lined in part with epithelium, there was considerable inflammation adjacent to the new gingival sulcus, and the diffuse inflammatory reaction extended to the alveolar crest. Some of the cells close to the sulcus were polymorphonuclear leukocytes, but most of the inflammatory cells were lymphocytes. The connective tissue of the newly formed marginal gingiva contained more collagen bundles than the shorter term specimens, but no collagen bundles were seen adjacent to the sulcular epithelium.

Fourteen days after the gingivectomy there were no more osteoclasts at the alveolar crest and a well defined osteoblastic layer had formed. The sulcus appeared in most sections to be lined by epithelium but a large number of lymphocytes and polymorphonuclear leukocytes were still present in occasional areas of incomplete epithelial coverage. There was an increase of collagen bundles in the free gingiva and the capillaries were small but often surrounded by lymphocytes. Within the periodontal membrane several endothelial cells were synthesizing DNA.

Twenty-one days following the gingivectomy there still was considerable diffuse

inflammation corresponding to the newly epithelialized gingival sulcus (Fig. 9A). There were a number of labeled cells adjacent to the sulcus wall and since many of them were small round cells, morphologically resembling lymphocytes, it was sometimes difficult to decide whether these cells were fibroblasts or lymphocytes. At this time there were still present a few polymorphonuclear leukocytes which were unlabeled. In the rest of the periodontium the vascularity had decreased compared to the earlier specimens, and the connective tissue was well organized in distinct collagen bundles. An occasional fibroblast and endothelial cell were labeled in the connective tissue of the marginal gingiva. The cementoblasts were still irregular and partially absent for as much as 0.5 mm apically to the bottom of the new gingival sulcus (Fig. 9B).

In the specimens obtained 35 days following the gingivectomy, the epithelialization of the marginal gingiva and sulcus was complete and the connective tissue of the marginal gingiva was indistinguishable from the connective tissue of the untreated control areas. A few labeled endothelial cells and fibroblasts were occasionally observed in the newly formed free gingiva. At the coronal aspect the epithelial attachment (corresponding to the bottom of the open

gingival sulcus) there were some inflammatory cells, mainly lymphocytes and plasma cells (Fig. 10). These cells were unlabeled, but in this area of mild inflammatory reaction a few labeled endothelial cells and fibroblasts could be seen.

DISCUSSION AND SIGNIFICANCE

In contrast to the reported 2 days delay of inflammation following gingivectomy^{5, 6} an acute inflammatory reaction in the connective tissues underlying the gingivectomy wound was observed 2 hours following the surgery. This initial reaction was mainly an emigration of polymorphonuclear leukocytes into the zone of tissue destruction resulting from the surgery. By 25 hours the connective tissue underlying the wound surface could be separated into a "poly-band" as described in rabbits by Viziam, et al.,⁷ and a deeper zone of apparently intact connective tissues. Some investigators^{8, 9} have described an acute inflammatory reaction as early as 15 to 20 minutes following injury. It appears from the present investigation that the acute inflammatory reaction after gingivectomy in monkeys follows the same pattern as has been reported for skin wounds^{7, 8} of other animals. Since the healing sequences have been found to be essentially the same in humans and animals, it seems likely that this early inflammatory reaction also is present in humans.

The "poly-band"⁷ involved both the superficial necrotic and degenerated connective tissue and part of the surface blood clot. It appears that this "poly-band" serves a protective function against bacterial invasion until the regenerating epithelium covers the wound. The migrating epithelial cells seem to wedge their way under the "poly-band" between the necrotic and the underlying viable connective tissue. Thus the new epithelium secures metabolic support from living connective tissue. The exact relationship between the migrating epithelium, the organization of the basement membrane, and the restitution or new formation of collagen at the contact areas



Fig. 10. Restored epithelial attachment and collagenous gingiva in 35 day specimen. (Magnification X87).

between the regenerating epithelium and the connective tissue cannot be determined from this study.

The almost complete disappearance of polymorphonuclear cells as soon as the wound surface was protected by epithelium is in accord with current concepts of a very short life span for these cells.¹⁰ The lymphocytes, with a much longer life span, prevailed for several weeks after the healing of the wound.

Obviously, the most critical phase of healing of the gingivectomy wound is the establishment of what appears to be a bacteria proof seal between the regenerating epithelium and the calcified surface of the tooth. In this area polymorphonuclear cells were present until a normal gingival sulcus was established 35 days after the gingivectomy. It can be assumed that if oral bacteria or their toxins had access to the connective tissue, there would have been an inflammatory reaction present. Even at that time some lymphocytes and plasma cells could be observed corresponding to the coronal aspect of the new epithelial attachment.

The connective tissue proliferation in the healing gingivectomy wound started later than the epithelial proliferation and followed a pattern that previously had been reported for other types of wounds, but had not been described for gingivectomy wounds.^{11, 12} The first definite increase in labeling of connective tissue cells occurred 24 hours after the surgery, within the supracrestal tissues, 0.3 to 0.5 mm from the wound surface. In this area of maximum cellular activity the average counts of labeled cells postoperatively were: 1 day—11.4 $\sqrt{\text{mm}^2}$; 2 days—35.6 $\sqrt{\text{mm}^2}$; 3 days—64.2 $\sqrt{\text{mm}^2}$; 5 days—49.5 $\sqrt{\text{mm}^2}$; and 7 days—39.0 $\sqrt{\text{mm}^2}$.

After 7 days there were no localized concentrations of cell proliferation. It appeared that the peak premitotic activity in the connective tissues was reached 3 days postoperatively, while for the epithelium the peak was reached 24 to 36 hours after the surgery.

Another important observation is that the connective tissue proliferation was initiated away from the very margin of the wound, but as soon as the wound was covered by epithelium, connective tissue proliferation also was assumed by cells immediately under the basement membrane of the epithelium and spread over the total supracrestal tissues.

The proliferative activity from 3 to 7 days produced a vascular granulation tissue which formed the new free gingiva. The governing stimulus for this regeneration of the free gingiva is probably related to functional demand and the anatomical form of the adjacent structures. Collagen formation and maturation developed gradually over 3-4 weeks as the inflammation and vascularity decreased. It took between 21 and 35 days before the synthesis of DNA and labeling of the total gingival tissues reached the control level of nonsurgerized normal gingiva. This indicates that it takes between 3 and 5 weeks for a simple gingivectomy wound on the buccal or lingual surfaces of

the teeth of a rhesus monkey to heal and regain functional properties although the gingival surface appears completely healed 1 to 2 weeks after the surgery.

Several recent studies on the origin of the fibroblast in the healing wound have stressed that the predominant number of fibroblasts in granulation tissue arise locally from perivascular regions.^{11, 13, 14} The marked synthesis of DNA observed in the perivascular areas of the gingiva supports this concept. According to Grillo¹³ the fibroblast-capillary system may be regarded as the primary reparative system in the mammal with the exception of the epithelial surfacing, this is the system which accomplishes restoration of form and function after injury. It is not yet possible to state whether the cells which respond to proliferative stimuli are differentiated fibroblasts or dormant mesenchymal cells which modulate to fibroblasts in response to injury.

The great number of endothelial cells undergoing synthesis of DNA and labeling during the first week of healing of the gingiva provides further evidence that the endothelial cells of the healing wound proliferate.^{15, 16} This concept has been questioned by other investigators.^{17, 18}

It cannot be definitely stated why so many of the osteoblasts on the outer aspect of the alveolar crest disappeared within 13 hours after the surgery, and why several days elapsed before a normal arrangement appeared. Klingsberg and Butcher¹⁹ reported an even more severe reaction both in the connective tissues and osteoblasts following removal of palatal gingiva in gingivectomy wounds in rats. The disruption of the crestal osteoblasts may be the result of the wounding as concluded by Klingsberg and Butcher.¹⁹ A few labeled osteoblasts started to appear at the alveolar crest 5 days postoperatively, and there was evidence of some remodeling of the alveolar crest in the 7 and 9 day specimens, possibly as a result of changes in functional demands associated with reestablishment of

gingival function. This process appeared to be completed after 14 days.

The initial disappearance of cementoblasts close to the wound after 5 hours is a dramatic demonstration of how sensitive these cells are to metabolic changes. Labeled cementoblasts were observed in longer term specimens as part of the completion of healing. Labeled Malassez's epithelial rests demonstrated the proliferative potential of these structures when stimulated.

Probably the most significant observation of this investigation is that complete healing of a gingivectomy wound with restoration of periodontal health is not completed until the gingival fibers have established their functional arrangement and collagen maturity, and thereby provide the basis for establishment and maintenance of a sealing epithelial attachment and a physiologic gingival crevice.

SUMMARY

The connective tissue aspect of healing and regeneration following simple gingivectomy was studied in three monkeys with histologic and radioautographic techniques. Acute inflammation was observed 2 hours after the surgery. The connective tissue labeling started later than the epithelial labeling and reached a peak at the 3rd day. The epithelial labeling reached a peak 1 day after the surgery. The connective tissue healing started 0.3 to 0.5 mm under the wound surface, but spread to the rest of the supracrestal tissues after epithelialization of the wound. It took between 21 and 35 days for complete healing of the gingivectomy wound and restoration of functional gingival health.

CONCLUSIONS

The following conclusions apply to healing of simple gingivectomy wounds in adult rhesus monkeys:

1. Connective tissue healing starts 0.3

to 0.5 mm under the "poly-band" protective surface.

2. After the surface has epithelialized, the connective tissue proliferation involves all of the supracrestal tissues up to the basement membrane of the new epithelium.
3. The connective tissue proliferation is initiated 1 to 2 days after the surgery and reaches a peak 3 to 4 days after the surgery.
4. Most of the regeneration of the free gingiva takes place from the 3rd to the 9th day after the surgery, and initially consists of vascular granulation tissue.
5. Functional arrangement and collagenous maturation of the gingival connective tissue fibers require 3 to 5 weeks.
6. A physiologic gingival crevice, with a sealing normal epithelial attachment, is dependent on a firm gingival tone which requires 3 to 5 weeks to establish following gingivectomy.

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RAOUL HENRY BLANQUIE, D.D.S.



Dr. Blanquie died unexpectedly Saturday, February 26, 1966, aged 70 years. A native of San Francisco, he had lived and practiced in Oakland, limiting to periodontia in recent years. He graduated from the School of Dentistry of the College of Physicians and Surgeons in 1917.

Dr. Blanquie was active in many organizations. He was past-president of the San Francisco District Dental Society, the California State Dental Association, the Alumni Association of the College of Physicians and Surgeons and the California Academy of Periodontology. He was General Chairman of the American Dental Association meeting held in San Francisco in 1936 and was a Past Vice-president of the American Dental Association.

His particular dental interest was periodontics. He was one of the early followers of Dr. A. W. Ward, and introduced the teaching of periodontal surgery into the undergraduate curriculum at the College of Physicians and Surgeons in 1925. He became Associate Professor of Periodontia at Physicians and Surgeons and later was visiting lecturer at the University of California.

Dr. Blanquie had given many clinics in the United States as well as France and Venezuela. He has written extensively on various aspects of periodontia with over thirty published articles.

Dr. Blanquie was a member of the Psi Omega dental fraternity, a Fellow of the American College of Dentists and the Tau Kappa Omega and Omicron Kappa Upsilon honor societies. He was also a member of the American Academy of Periodontology and the Northern California Society of Periodontists.

Dr. Blanquie was a leader of the French colony here and was a past president of the Alliance Francaise and an active member of the Cercle de l'Union. For his services to the French community, Dr. Blanquie held the rank of officer in the Legion of Honor from the French Government. A noted gourmet and connoisseur of wines, he was president of the Wine and Food Society of San Francisco.

Dr. Blanquie is survived by his wife Jeanne, two daughters, Vivienne and Mrs. Marshall Moran, and seven grandchildren.

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