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TRITIATED THYMIDINE AUTORADIOGRAPHIC STUDY ON THE INFLUENCE OF SENSORY AND SYMPATHETIC INNERVATION ON PERIODONTAL WOUND HEALING IN THE RAT

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Summary—Understanding of wound healing mechanisms is important in designing preventive and therapeutic approaches to inflammatory periodontal diseases, which are a major cause of dental morbidity. In this study, cell proliferation was assessed after an experimental gingival wound; this was preceded by either resection of 3 mm of the inferior alveolar nerve, total extirpation of the superior cervical ganglion, trauma to those structures or sham operations. At different times, animals were pulsed with 0.5 μCi/g body weight of tritiated thymidine; histological sections were processed for quantitative autoradiography of different compartments of the peridontium. Wounding led to a significant increase in cell proliferation in the epithelial layer, the fibroblast compartment and the periodontal ligament, but not in the alveolar crest compartment. Sympathetic denervation significantly enhanced this response in the epithelial layer, the fibroblast compartment and the alveolar crest, whereas sensory denervation only modified the response in the fibroblast layer. Thus it appears that sympathetic innervation plays an important role in the regulation of cell proliferation in the periodontium and that pharmacological modulation of sympathetic activity should be further studied as a therapeutic approach in periodontal disease.

Key words: wound healing, periodontal disease, sympathetic innervation, autoradiography.

INTRODUCTION

Over the past decade periodontitis has become a major cause of dental morbidity as fluoridation and oral hygiene education have succeeded in controlling caries. Despite the good healing capacity of the periodontium (Melcher, 1976), there is a high incidence of poorly healing chronic lesions.

The mechanisms controlling regeneration of the periodontium are not fully understood. It would be of particular interest to advance our understanding of how a balance is maintained among the epithelial and connective tissues of the periodontium. The nervous system may play an important role in coordinating growth and differentiation in normal and inflamed gingiva. Several studies have suggested a role for the peripheral nervous system in controlling the metabolism and proliferation of osteoblasts (Aro *et al.*, 1981; Singh *et al.*, 1982), odontoblasts (Avery, Cox and Corpron, 1974; Chiego, 1984) and also rabbit corneal epithelium (Chan *et al.*, 1987). We have undertaken a quantitative autoradiographic study of the influence of sensory and sympathetic denervation on the coordinated response of epithelial cells, fibroblasts and bone-related cells to gingival injury.

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Abbreviations: IAN, inferior alveolar nerve; SCG, superior cervical ganglion.

MATERIALS AND METHODS

Experimental procedures

One hundred and twenty-six Sprague–Dawley rats (200–250 g) were divided into 7 groups of 18 animals each. They were fed rodent laboratory chow (Purina Mills, Inc.) and water *ad libitum* and were kept in a 12 h light–dark cycle throughout the experiment.

The experimental gingival wound was made by removing the mesiobuccal gingival papilla of the lower right first molar by brief application of electrocautery. The alveolar bone was not exposed. Before this, resection of the IAN, extirpation of the SCG, traumatization of either nerve structure or respective sham operations were performed as described below. Surgery was done under sodium pentobarbital anaesthesia (5 mg/100 g body weight). The following operations were performed in different groups.

1. Sensory denervation by resection of approx. 3 mm of the right IAN at the distal end of the incisor, as described by Chiego and Singh (1981). In brief, an incision was made in skin overlying the masseter muscle; access to the mandibular bone was obtained by blunt dissection of the muscle fibres; the mandibular canal was opened carefully with a curette, the nerve lifted up with a pair of forceps and 3 mm of nerve removed (=IAN RE).

2. Traumatization of the IAN at the same site, consisting of exposure as described above and lifting the nerve with a pair of forceps (=IAN TR).

3. Sham operation at the same site; the mandibular canal was exposed but not opened (=IAN SE).

4. Sympathetic denervation by extirpation of the right SCG, as described by Avery *et al.* (1971). In initial experiments the excised ganglion was paraffin-embedded and identified by light microscopy. After recovery (=SCG RE) the right and left pupil diameters were compared to evaluate the success of the operation.

5. The ganglion was traumatized by exposure of the SCG lying underneath the carotid bifurcation (=SCG TR).

6. Sham operation consisting of skin incision and blunt dissection of superficial cervical muscles only (=SCG SE).

7. No surgical procedure performed other than the experimental periodontal wound (=WOUND E).

The following abbreviations—WOUND C, IAN RC, IAN TC, IAN SC, SCG RC, SCG TC and SCG SC—refer to respective contralateral controls.

Three rats per group were killed at 6, 12, 24 h and 3, 6, 12 days after periodontal wounding. One hour before death, the animals were injected intraperitoneally with 0.5 μCi/g body weight [^{3}H]-thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear Co., Boston, MA, U.S.A.). This schedule facilitates labelling of cells subsequently entering mitosis, thus allowing an assessment of the proliferative activity (Tonna and Cronkite, 1962).

Histological processing

After perfusion with phosphate-buffered saline and 4% paraformaldehyde the mandibles were removed, postfixed for 48 h, washed and demineralized in 0.5 mol/l EDTA (monitored by X-ray films). The tissue was dehydrated and paraffin-embedded. Specimens were orientated to obtain cross-sections of the mandible showing tooth crown and root, alveolar bone, buccal and lingual gingiva (Fig. 1). Sections (5 μm) were mounted on glass slides, deparaffinized, dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY, U.S.A.) and exposed for 14 days in light-tight boxes at 4°C. Slides were developed in Kodak D-19 developer and rapid fix, washed and counterstained with Harris' haematoxylin and eosin.

Autoradiographic and statistical analysis

To facilitate analysis of cell proliferation, the labelling index was determined in four different compartments (modified after Tonna and Stahl, 1973) adjacent to the wound site (Fig. 1):

I. Cells in the basal layer of the gingival epithelium.
II. Fibroblasts in the connective tissue below the attached gingiva.
III. Cells at the alveolar crest.
IV. Bone-related cells in the periodontal ligament.

Corresponding areas were counted on the intact contralateral side. All cells with more than three grains were considered positive. Three sections on three different slides were evaluated per specimen and the labelling index determined. According to Tonna, Stahl and Slywka (1977) the labelling index is the ratio of tritiated thymidine-labelled cells to the total cell population count for the compartment under study. The mean, standard deviation and standard error of the mean were calculated for each group. Paired t-tests were used to compare the experimental and intact control side in each group, and unpaired t-tests to compare the experimental and sham experimental groups (1–6) with the wound experiment group (WOUND E, group 7).

RESULTS

Gingival wound healing followed the same pattern as described by Engler, Ramfjord and Hiniker (1966) after gingivectomy with a scalpel. This included oedema after 6 h accompanied by intense infiltration of polymorphonuclear cells in the wound site and acute inflammatory reactions after 12 h. Three to six days after wounding, epithelium had covered the site. After 6 days, the beginning of differentiation of the gingival papilla and a sulcus at the tooth surface was visible. At 12 days the area had an almost regular appearance, but the papilla had not reached normal height.

Different cell types showed different baseline mitotic activity as assessed on the intact contralateral side. The labelling index was highest in the basal layer of the epithelium, reaching values of up to 13% labelled cells (Table 1), and ranged between 1 and 2–5% for fibroblasts and cells of the alveolar crest and periodontal ligament, respectively (Tables 2, 3, 4).

In most of the cell compartments, nerve resection and trauma to the nervous structure led to higher labelling indices after wounding. In contrast, sham operations that left nervous structures intact gave results similar to those from groups in which only gingival wounding was performed.

SCG extirpation had a profound influence on cell proliferation in the periodontium after wounding. Labelling indices were significantly higher in the epithelial cell layer, as well as in the fibroblast compartment and the alveolar crest when compared to the group that had gingival wounding alone. Similar, but less pronounced changes were seen with SCG trauma. In contrast, IAN resection had a smaller overall effect on cell proliferation in the periodontium. Significant changes occurred in the fibroblast compartment only; the few changes in the alveolar crest and periodontal ligament were not statistically significant.

In the epithelial layer, proliferative activity rose from 5% labelled cells to 14.8% at 24 h after wounding (Table 1). Prior removal of the SCG

Plate 1

Fig. 1. Photomicrograph of the areas examined. I—Epithelial cell compartment. II—Fibroblast compartment. III—Cells at the alveolar crest. IV—Bone-related cells in the periodontal ligament. H&E, ×80.

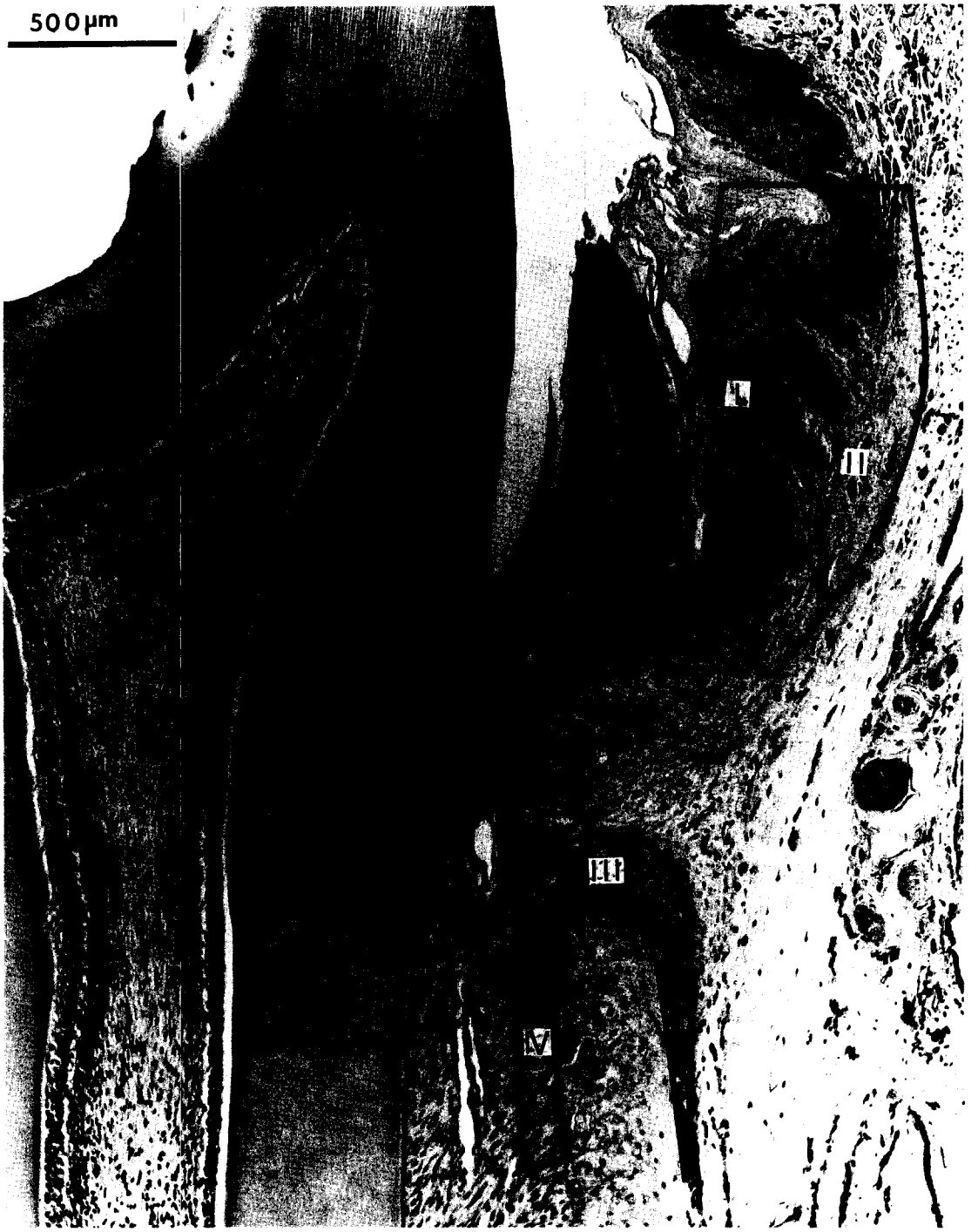

Plate 1

Table 1. Labelling index (mean ± SEM) of cells in the basal layer of the epithelium adjacent to the wound site. 6, 12, 24 h and 3, 6, 12 days after nerve resection and/or wounding

	6 hours	12 hours	24 hours	3 days	6 days	12 days
IAN RE	0.087 ± 0.039	0.091 ± 0.010	0.228 ± 0.032	0.156 ± 0.048	0.139 ± 0.008	0.094 ± 0.026
Control side	0.062 ± 0.021	0.073 ± 0.017	0.095 ± 0.005	0.127 ± 0.026	0.082 ± 0.016	0.088 ± 0.008
IAN TR	0.030 ± 0.0	0.113 ± 0.0*	0.235 ± 0.030*	0.096 ± 0.014†	0.057 ± 0.007	0.045 ± 0.006†
Control side	0.033 ± 0.009	0.066 ± 0.009	0.116 ± 0.011	0.072 ± 0.015	0.074 ± 0.11	0.055 ± 0.012
IAN SE	0.122 ± 0.019	0.082 ± 0.012	0.203 ± 0.025	0.201 ± 0.043*	0.075 ± 0.013	0.059 ± 0.013
Control side	0.080 ± 0.007	0.040 ± 0.009	0.121 ± 0.010	0.098 ± 0.33	0.072 ± 0.015	0.041 ± 0.002
WOUND E	0.063 ± 0.021	0.106 ± 0.035	0.148 ± 0.029	0.213 ± 0.015*	0.095 ± 0.039	0.083 ± 0.11
Control side	0.044 ± 0.014	0.065 ± 0.008	0.085 ± 0.005	0.130 ± 0.016	0.074 ± 0.011	0.079 ± 0.008
SCG RE	0.035 ± 0.011	0.141 ± 0.032	0.377 ± 0.069†	0.344 ± 0.041*†	0.228 ± 0.084	0.109 ± 0.031
Control side	0.043 ± 0.019	0.048 ± 0.011	0.243 ± 0.049	0.172 ± 0.026	0.196 ± 0.097	0.074 ± 0.023
SCG TR	0.061 ± 0.007	0.215 ± 0.026*	0.260 ± 0.011†	0.217 ± 0.031	0.159 ± 0.076	0.145 ± 0.011†
Control side	0.049 ± 0.007	0.087 ± 0.018	0.175 ± 0.043	0.109 ± 0.010	0.094 ± 0.008	0.129 ± 0.016
SCG SE	0.036 ± 0.0*	0.080 ± 0.012	0.150 ± 0.029	0.186 ± 0.023	0.044 ± 0.003	0.085 ± 0.009
Control side	0.028 ± 0.001	0.051 ± 0.005	0.095 ± 0.006	0.039 ± 0.006	0.052 ± 0.016	0.075 ± 0.006

*$p < 0.05$ for paired comparison of experimental versus control side.
†$p < 0.05$ for unpaired comparison of experimental versus WOUND E.

Table 2. Labelling index (mean ± SEM) of fibroblasts adjacent to the wounded area. 6, 12, 24 h and 3, 6, 12 days after nerve resection and/or wounding

	6 hours	12 hours	24 hours	3 days	6 days	12 days
IAN RE	0.008 ± 0.003	0.005 ± 0.001	0.014 ± 0.002	0.155 ± 0.004*†	0.078 ± 0.029	0.011 ± 0.004
Control side	0.018 ± 0.008	0.004 ± 0.001	0.005 ± 0.002	0.008 ± 0.002	0.011 ± 0.006	0.011 ± 0.003
IAN TR	0.003 ± 0.001	0.004 ± 0.001	0.013 ± 0.003	0.161 ± 0.023*†	0.014 ± 0.005	0.014 ± 0.004*
Control side	0.004 ± 0.001	0.007 ± 0.002	0.009 ± 0.004	0.006 ± 0.001	0.007 ± 0.001	0.010 ± 0.004
IAN SE	0.004 ± 0.001	0.004 ± 0.001*	0.019 ± 0.004	0.100 ± 0.016*	0.046 ± 0.013	0.019 ± 0.005
Control side	0.005 ± 0.0	0.003 ± 0.001	0.004 ± 0.0	0.004 ± 0.001	0.005 ± 0.001	0.004 ± 0.0
WOUND E	0.002 ± 0.001	0.004 ± 0.0	0.007 ± 0.003	0.055 ± 0.008*	0.033 ± 0.011	0.026 ± 0.016
Control side	0.004 ± 0.001	0.005 ± 0.002	0.010 ± 0.002	0.008 ± 0.003	0.005 ± 0.0	0.006 ± 0.002
SCG RE	0.008 ± 0.003	0.004 ± 0.002	0.010 ± 0.006	0.126 ± 0.003*†	0.084 ± 0.003*†	0.007 ± 0.001
Control side	0.006 ± 0.0	0.006 ± 0.002	0.038 ± 0.019	0.013 ± 0.004	0.015 ± 0.004	0.010 ± 0.002
SCG TR	0.008 ± 0.002	0.035 ± 0.027	0.013 ± 0.004	0.115 ± 0.007*†	0.130 ± 0.060	0.022 ± 0.011
Control side	0.010 ± 0.001	0.012 ± 0.002	0.015 ± 0.006	0.020 ± 0.006	0.020 ± 0.006	0.012 ± 0.008
SCG SE	0.005 ± 0.001	0.006 ± 0.004*	0.005 ± 0.001*	0.084 ± 0.020	0.073 ± 0.026	0.028 ± 0.017
Control side	0.007 ± 0.002	0.007 ± 0.001	0.008 ± 0.003	0.011 ± 0.005	0.008 ± 0.002	0.011 ± 0.003

*$p < 0.05$ for paired comparison of experimental versus control side.
†$p < 0.05$ for unpaired comparison of experimental versus WOUND E.

Table 3. Labelling index (mean ± SEM) of cells at the alveolar crest adjacent to the wound site. 6, 12, 24 h and 3, 6, 12 days after nerve resection and/or wounding

	6 hours	12 hours	24 hours	3 days	6 days	12 days
IAN RE	0.011 ± 0.010	0.032 ± 0.003	0.048 ± 0.018	0.067 ± 0.026	0.015 ± 0.003	0.014 ± 0.002
Control side	0.010 ± 0.001	0.026 ± 0.010	0.02 ± 10.002	0.010 ± 0.001	0.014 ± 0.006	0.005 ± 0.002
IAN TR	0.016 ± 0.012	0.010 ± 0.005*	0.056 ± 0.022	0.033 ± 0.016	0.008 ± 0.006	0.008 ± 0.005
Control side	0.013 ± 0.003	0.032 ± 0.006	0.025 ± 0.007	0.011 ± 0.001	0.013 ± 0.006	0.012 ± 0.004
IAN SE	0.019 ± 0.015	0.008 ± 0.004	0.023 ± 0.014	0.098 ± 0.008*†	0.060 ± 0.012*	0.016 ± 0.005
Control side	0.040 ± 0.014	0.020 ± 0.003	0.031 ± 0.008	0.014 ± 0.003	0.015 ± 0.004	0.013 ± 0.003
WOUND E	0.015 ± 0.007*	0.015 ± 0.006	0.024 ± 0.012	0.052 ± 0.007	0.047 ± 0.019	0.014 ± 0.001*
Control side	0.025 ± 0.004	0.026 ± 0.005	0.026 ± 0.007	0.024 ± 0.010	0.015 ± 0.002	0.008 ± 0.001
SCG RE	0.004 ± 0.002	0.003 ± 0.003	0.128 ± 0.041	0.207 ± 0.021*†	0.043 ± 0.023	0.011 ± 0.003
Control side	0.018 ± 0.006	0.010 ± 0.006	0.057 ± 0.011	0.043 ± 0.003	0.031 ± 0.015	0.014 ± 0.006
SCG TR	0.008 ± 0.005	0.020 ± 0.017	0.072 ± 0.039	0.125 ± 0.021†	0.047 ± 0.020	0.008 ± 0.002†
Control side	0.013 ± 0.003	0.029 ± 0.010	0.040 ± 0.009	0.024 ± 0.011	0.012 ± 0.002	0.016 ± 0.004
SCG SE	0.002 ± 0.0	0.009 ± 0.009	0.069 ± 0.033	0.096 ± 0.022	0.030 ± 0.008	0.017 ± 0.0†
Control side	0.006 ± 0.002	0.042 ± 0.010	0.030 ± 0.004	0.011 ± 0.0	0.006 ± 0.002	0.014 ± 0.003

*$p < 0.05$ for paired comparison of experimental versus control side.
†$p < 0.05$ for unpaired comparison of experimental versus WOUND E.

enhanced this response to 37%, which indicates that at the time point of [^{3}H]-thymidine pulsing more than a third of the cells in the epithelial layer had progressed into the S-phase of the cycle. In addition, a more rapid increase in labelling was apparent after SCG resection, as peak proliferation occurred at 24 h in the resected (37.7%) as compared to 3 days in the non-resected animals (21.3%). Similar but smaller changes were found after IAN resection and IAN trauma.

Table 4. Labelling index (mean ± SEM) of bone-related cells in the periodontal ligament 6, 12, 24 h and 3, 6, 12 days after nerve resection and/or wounding

	6 hours	12 hours	24 hours	3 days	6 days	12 days
IAN RE	0.013 ± 0.005	0.012 ± 0.0†	0.022 ± 0.014	0.042 ± 0.016	0.030 ± 0.015	0.028 ± 0.004*
Control side	0.026 ± 0.002	0.019 ± 0.008	0.027 ± 0.003	0.043 ± 0.008	0.023 ± 0.003	0.047 ± 0.002
IAN TR	0.018 ± 0.005*	0.011 ± 0.005	0.011 ± 0.006	0.023 ± 0.002†	0.006 ± 0.004*†	0.026 ± 0.007
Control side	0.025 ± 0.005	0.026 ± 0.006	0.021 ± 0.004	0.018 ± 0.003	0.017 ± 0.003	0.032 ± 0.005
IAN SE	0.020 ± 0.009	0.043 ± 0.021	0.004 ± 0.001*†	0.081 ± 0.017*	0.037 ± 0.008	0.040 ± 0.005
Control side	0.013 ± 0.004	0.021 ± 0.002	0.074 ± 0.012	0.050 ± 0.017	0.047 ± 0.019	0.042 ± 0.012
WOUND E	0.010 ± 0.001	0.020 ± 0.001	0.037 ± 0.012	0.076 ± 0.005*	0.042 ± 0.007	0.048 ± 0.009
Control side	0.008 ± 0.004	0.026 ± 0.004	0.034 ± 0.005	0.037 ± 0.007	0.043 ± 0.017	0.018 ± 0.004
SCG RE	0.017 ± 0.012	0.014 ± 0.008	0.046 ± 0.006	0.116 ± 0.028	0.056 ± 0.010	0.041 ± 0.009
Control side	0.029 ± 0.004	0.018 ± 0.007	0.073 ± 0.017	0.074 ± 0.017	0.053 ± 0.008	0.035 ± 0.013
SCG TR	0.032 ± 0.012	0.034 ± 0.025	0.010 ± 0.005	0.134 ± 0.023	0.045 ± 0.017	0.042 ± 0.015
Control side	0.021 ± 0.011	0.020 ± 0.013	0.066 ± 0.016	0.042 ± 0.008	0.056 ± 0.005	0.037 ± 0.007
SCG SE	0.019 ± 0.001†	0.016 ± 0.006	0.013 ± 0.004	0.076 ± 0.013*	0.032 ± 0.014	0.039 ± 0.009
Control side	0.012 ± 0.0	0.020 ± 0.005	0.039 ± 0.008	0.028 ± 0.004	0.029 ± 0.006	0.042 ± 0.007

*$p < 0.05$ for paired comparison of experimental versus control side.
†$p < 0.05$ for unpaired comparison of experimental versus WOUND E.

In the fibroblast compartment, the labelling index reached its peak values (5.5%) at day 3 after gingival wounding alone. Both SCG and IAN operations (resection or trauma) resulted in significant increases in cell proliferation (IAN RE 5.5%, SCG RE 12.6%), with SCG and IAN resections giving similar results (Table 2).

SCG resection resulted in a major increase in labelling in the alveolar crest compartment at 24 h (12.8%) and 3 days (20.7%), but IAN resection had only a minor effect (4.8 and 6.7% respectively). The labelling index rose significantly from the 2.5% baseline to 12.8% and 20.7% at 24 and 72 h after wounding; only 2.4% (24 h) and 5.2% (3 days) of cells were labelled in the non-resected group. Again, the SCG trauma produced a significant response that was smaller than in the SCG resected animals (Table 3).

Similar observations were made in the periodontal ligament, but the values did not reach statistical significance (Table 4).

DISCUSSION

We have studied the influence of sympathetic and sensory denervation on wound healing in rat gingiva by quantitative autoradiography. The labelling indices found after wounding alone correlate in their magnitude and time course with those found during gingival repair by other investigators (Engler *et al.*, 1966; Stahl, Tonna and Weiss, 1968; Tonna, Stahl and Weiss, 1969).

Sympathetic ganglionectomy significantly increased DNA synthesis in the regenerating periodontium and had a major effect on the proliferation of epithelial cells, fibroblasts and cells of the alveolar crest. In contrast, no major effect was found in the periodontal ligament, which may be due to a local, nerve-independent regulation of proliferation and cell death, as suggested by McCulloch, Barghava and Melcher (1989). The effect observed in the other compartments had an early peak at 24–72 h after wounding. Damaging the ganglionic structure had a smaller, but similar effect; sham operations did not produce significantly different effects from those in animals that had received gingival wounds only. These findings support the interpretation that the differences in cell proliferation were due to interference with the functional activity of the sympathetic ganglion.

The sympathetic neurones from the superior cervical ganglion extensively innervate the head and neck. The thin, unmyelinated axons travel with the arterial vasculature and branch extensively in their target structures. Studies of the rat iris have demonstrated numerous axonal varicosities containing vesicles which are in direct contact with innervated smooth muscle cells and which are thought to have synaptic functions (Olson and Malmfors, 1970). A major effect of cervical ganglionectomy is a rapid vasodilatation in the head and neck, which has been observed after therapeutic ganglionectomy for Raynaud's disease in humans (Brodal, 1981).

Potential mechanisms for the observed effect of sympathetic denervation on cell proliferation in the healing gingiva are as follows.

1. Vasodilatation in the head and neck, with a resulting increase in blood flow, may lead to an increased supply of nutrients and blood-derived growth factors, such as epidermal growth factor, fibroblast growth factor, platelet-derived growth factor and insulin (Ross and Vogel, 1978).
2. There may be a local increase in the production of neurotrophic growth factors after gingival denervation, which may also have a mitogenic effect on periodontal cells such as epithelium and fibroblasts. Receptors for nerve growth factor have been demonstrated on non-neuronal cells such as keratinocytes (Thomson *et al.*, 1988).
3. The resection may remove a physiological, suppressive effect of the sympathetic nervous system on gingival cell proliferation. The increase in epithelial proliferation on the contralateral, intact side demonstrates that such activity after ganglionectomy also occurs in the unwounded gingiva. This may be interpreted as evidence for a suppressive effect of the SCG on cell proliferation in the unwounded gingiva.

The increase in cell proliferation in keratinocytes, fibroblasts and cells of the alveolar crest 24–72 h after denervation or injury does not point to the sole presence of a single, highly specific factor, but suggests that a number of different factors and/or a broadly reactive factor are responsible for the observed phenomena.

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