Acceleration of Wound Healing With Topically Applied Deoxyribonucleosides

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Hypothesis: We hypothesized that a topical mixture of purified deoxyribonucleosides would accelerate wound healing in an open wound model.

Design: Full-thickness 6-mm wounds were made on the ears of young adult rabbits. In some experiments, 2 of the 3 arteries in each ear were divided to induce wound ischemia.

Interventions: An equiweight mixture containing all 4 of the major deoxyribonucleosides (deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine), designated PN105, or other subgroups of deoxyribonucleosides, or vehicle (saline) was applied to wounds on 1 ear every 2 days, with the other ear serving as a control.

Main Outcome Measures: Wound tissue was processed for histological examination 7 days after the initial wounding. Granulation tissue formation and epithelialization were measured in histological cross sections of wounds.

Results: Treatment of wounds with PN105 resulted in a 191% increase in total new granulation tissue (P<.05) and a higher incidence of complete wound reepithelialization (67% vs 37%; P<.05) when compared with controls, and a similar increase under ischemic conditions on day 7. Wound ischemia markedly impairs healing; PN105 treatment resulted in a 242% increase in the amount of new granulation tissue formed by day 7 in ischemic wounds, relative to the appropriate controls (P<.05). All 4 of the major deoxyribonucleosides were required for optimum activity; mixtures with 3 or 2 were less active or inactive.

Conclusions: Topically applied deoxyribonucleosides reproducibly accelerate wound healing in normal and ischemic wounds, and to a magnitude equivalent to that of recombinant growth factors such as platelet-derived growth factor, previously studied in this model. In view of their safety, availability, and efficacy, deoxyribonucleosides hold considerable promise for improving healing of chronic wounds.

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MATERIALS AND METHODS

The rabbit ear dermal ulcer model developed by Ahn and Mustoe and Mustoe and colleagues was used for evaluation of wound healing activity of test compounds. This quantitative wound healing model has been validated and extensively used for testing the relative and absolute activities of a variety of putative wound healing factors. Young adult female New Zealand white rabbits (Hazelton-Dutchland, Inc, Denver, Pa), weighing 2.7 to 3.4 kg were acclimated and housed under standard conditions, per experimental protocol approved by the Northwestern University Animal Care and Use Committee (Chicago, Ill).

Surgery was performed under sterile conditions after rabbits were anesthetized with ketamine hydrochloride, 60 mg/kg (Sigma-Aldrich Corp, St Louis, Mo) and xylazine hydrochloride, 5 mg/kg (Sigma-Aldrich Corp). Three or 4 full-thickness (to bare cartilage) excisional wounds were made on the interior surface of each ear with a 6-mm tissue punch. Immediately after surgery (day 0), and at days 2 and 4, mixtures of nucleosides (each nucleoside at 10 mg/mL) in physiological saline (Pro-Neuron Inc, Gaithersburg, Md) or vehicle (sterile saline) were applied to wounds in a volume of 10 µL. Wounds were then covered with an occlusive dressing (Tegaderm; 3M Corp, Minneapolis, Minn). In some experiments, persistent ischemia was produced in rabbit ears before creation of the circular excision wounds by dividing the central and rostral arteries and dermal circulation. On day 7 after wounding, the animals were killed and the wounds were fixed in 10% formalin.

RESULTS

EFFECTS OF PN105 ON WOUND HEALING IN THE NORMAL RABBIT EAR ULCER MODEL

Complete epithelial closure (epithelial gap, 0 µm) was documented in 12 (67%) of the 18 PN105-treated wounds compared with only 7 (37%) of the 19 control wounds. The mean TNTV on day 7 was nearly 2-fold (191%) compared with only 7 (37%) of the 19 control wounds. Documented in 12 (67%) of the 18 PN105-treated wounds.

WOUND HEALING EFFECTS OF DIFFERENT MIXTURES OF DEOXYRIBONUCLEOSIDES IN THE RABBIT EAR ULCER MODEL

Earlier experimental work in rats indicated that the individual deoxyribonucleosides (deoxyadenosine [dA], deoxyguanosine [dG], deoxyxycytidine [dC], and thymidine [dT]) applied singly to full-thickness dermal excision wounds had no effect on wound healing. The key mixtures of 2 and 3 deoxyribonucleosides were tested and compared against the mixture of all 4 in the rabbit ear ulcer model. In the first study, the effects of PN105 were compared with those of dC + dG, dC + dG + dA, and dC + dG + dT. Each individual rabbit received treatment with PN105 (as a positive control) on 1 ear and 1 other mixture of deoxyribonucleosides on the other ear, to minimize effects of individual variation between animals. Treatment of wounds with PN105 resulted in significantly greater TNTV than any of the other tested mixtures of 2 or 3 of the constituent deoxyribonucleosides (Table 1). In the second study, PN105 was compared with the mixtures of dA + dT and dC + dG. The methods used were the same as those in the first mixture study. The results show that PN105 treatment produced greater TNTV than did either pair, although statistical significance was not reached (Table 2). However, when taken together with the results of Table 1 and previous work with a rat model, histological cross sections of wounds, stained with hematoxylin-eosin, were used for quantitative evaluation of wound healing by an observer blinded to treatment. Values for total new granulation tissue and new wound epithelium at 7 days were derived from measurements of (1) depth or height of new granulation tissue (P), (2) lateral distance from the wound margin covered by new granulation tissue (G), (3) distance between peaks of new granulation tissue (P-P), and (4) epithelial gap (EG). These variables were used to calculate the total new tissue volume (TNTV) by means of the following formula: $TNTV = \frac{1}{4}\pi \times SP \times TNG^2$, wherein SP represents superficial peaks ($P_1 + P_2$) and TNG is total new granulation tissue distance ($G_1 + G_2$). An alternative method of determining volume by a reticle method with a superimposed grid was also used for improved accuracy. Figure 1 shows these methods schematically. Values reported are means ± SEs.

The primary end point was total new tissue produced during the process of healing, expressed as TNTV or as the 2-dimensional area of new granulation tissue and epithelium in cross-sectional histological slides. An additional end point in some studies was the fraction of wounds completely reepithelialized at the time of tissue collection. In any given rabbit, only 1 pair of treatments was evaluated. Statistical analysis was performed with the Student t test for epithelial gap, granulation tissue gap, and peak of granulation tissue. The Fisher exact test was used for comparison of the incidences of complete epithelialization between groups, and 1-way analysis of variance was used for comparison of dose-response characteristics among rabbits.
the mixture of all 4 nucleosides was superior to any individual nucleoside and to any other mixture of nucleosides.

EFFECTS OF DEOXYRIBONUCLEOSIDES VS RIBONUCLEOSIDES ON WOUND HEALING IN THE NORMAL RABBIT EAR ULCER MODEL

Since, chemically, RNA is a linear polymer of ribonucleosides joined by phosphodiester moieties and DNA is composed of 2'-deoxyribonucleosides otherwise linked identically, the relative effects of a mixture of deoxyribonucleosides were compared with those of a corresponding mixture of ribonucleoside nucleosides (adenosine, cytidine, guanosine, and uridine), as a measure of the specificity of the deoxyribonucleoside combination.

For these experiments, a 4-mg/mL concentration of each deoxyribonucleoside or ribonucleoside was used rather than the 10 mg/mL used in previous experiments, because this represents the solubility limit of the ribonucleosides. The TNTV was significantly greater in the deoxyribonucleoside-treated wounds (n = 16) than in controls (n = 15); there was no statistically significant difference between ribonucleoside-treated wounds (n = 14) and their respective controls (Figure 2, B and D, and Figure 4). The deoxyribonucleosides at a concentration of 4 mg/mL in this experiment produced about the same improvement as they did at 10 mg/mL, as de-
scribed in the first experiment on normal wounds above (184% increase in new tissue at 4 mg/mL vs 191% at 10 mg/mL). The difference was not statistically significant.

EFFICACY OF A CARBOMER-BASED HYDROGEL FORMULATION OF PN105 IN THE RABBIT EAR ULCER WOUND HEALING MODEL

Control saline was demonstrated to have no effect on TNTV, and therefore PN105 (in the saline vehicle) was the active component in accelerating wound healing in the rabbit ear ulcer (Figure 2, A and B). For clinical use of PN105, a more viscous formulation with appropriate preservatives is preferable. Therefore, a carbomer-based hydrogel formulation was developed. The gel formulation contained each of the deoxyribonucleosides at 4 mg/mL, 0.625% Carbomer 934P (B. F. Goodrich Company, Cleveland, Ohio), 0.05% Quaternium-15 (Dow Chemical Company, Midland, Mich), 0.9% benzyl alcohol, and 0.05% EDTA in water (pH 6.9). A concentration of 4 mg/mL was selected on the basis of the observations that PN105 appears to be as effective at that dose as at the earlier tested concentration of 10 mg/mL and deoxyribonucleosides remain in solution in aqueous media at 4°C at 4 mg/mL but not at 10 mg/mL. Control wounds received either 0.9% saline alone or carbomer gel placebo. Sixteen wounds per group were evaluated except in the saline group used for comparison with the placebo gel group. In that case, 15 wounds were evaluable. Paired comparisons were made as before, with each ear being treated with 1 regimen, and thus 1 comparison being made with each group of rabbits. All permutations were tested, with the finding that the carbomer vehicle had no effect on healing, but that the PN105-carbomer combination was as effective as the PN105 in saline vehicle (Table 3). In addition, these separate experiments confirmed the efficacy of PN105 and demonstrated that they were not vehicle dependent.

### Table 1. Effects of PN105 and Mixtures of the Deoxyribonucleosides Containing dC and dG on TNTV*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNTV, mm³</th>
<th>dC + dG</th>
<th>dC + dG + dA</th>
<th>dC + dG + dT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN105</td>
<td>8.20 ± 0.49</td>
<td>5.23 ± 0.95</td>
<td></td>
<td></td>
<td>.006</td>
</tr>
<tr>
<td>PN105 + dA</td>
<td>8.08 ± 0.55</td>
<td>5.82 ± 0.81</td>
<td></td>
<td></td>
<td>.03</td>
</tr>
<tr>
<td>PN105 + dT</td>
<td>7.74 ± 0.65</td>
<td>4.80 ± 0.64</td>
<td></td>
<td></td>
<td>.008</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SEM. dC indicates deoxycytidine; dG, deoxyguanosine; TNTV, total new tissue volume; dA, deoxyadenosine; and dT, thymidine. PN105 is a mixture of the 4 deoxyribonucleosides.

### Table 2. Effects of PN105 and Other Mixtures of the Deoxyribonucleosides on TNTV*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNTV, mm³</th>
<th>dC ± dG</th>
<th>dA ± dT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN105</td>
<td>5.81 ± 0.62</td>
<td>4.70 ± 0.60</td>
<td></td>
<td>.20</td>
</tr>
<tr>
<td>PN105 + dA</td>
<td>5.24 ± 0.57</td>
<td>3.87 ± 0.48</td>
<td></td>
<td>.07</td>
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</tbody>
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*Data are given as mean ± SEM. See the footnote to Table 1 for explanation of abbreviations.
A mixture of the 4 major deoxyribonucleosides, designated PN105, accelerates granulation and epithelialization of full-thickness ulcers in rabbits when applied repeatedly (once every 2 days). A normal balance of glycosaminoglycans and collagen is present in granulation tissue produced under the influence of PN105. The wound-healing activity is specific to deoxyribonucleosides (ie, in contrast to ribonucleosides), and the best activity is obtained when all 4 deoxyribonucleosides are present in the formulation together. PN105 is active in ischemic wounds, unlike some polypeptide wound healing factors.\(^\text{16}\) PN105 is equally active in saline and in a hydrogel formulation. The activity of PN105 is comparable with that of platelet-derived growth factor or transforming growth factor \(\beta\) on total new wound healing tissue volume at day \(7.17\)

Earlier literature suggested positive effects of topically applied DNA on formation of granulation tissue in excisional wounds.\(^\text{18-20}\) The results presented herein support the validity of these results, and also suggest that the effect can be explained as a result of degradation of DNA to the level of deoxyribonucleosides (via the actions of ubiquitous nucleases and phosphatases), which are efficiently taken up by cells via carriers, in contrast to deoxynucleotides or oligodeoxyribonucleotides, which are restricted in their entry into cells by their anionic phosphate moieties.

When nucleosides are taken up by cells, they are rapidly phosphorylated to the level of nucleotides, which can then be used for nucleic acid synthesis or other purposes. It is generally assumed that deoxyribonucleotide availability is not normally limiting for cell proliferation, since cells have ribonucleotide reductase, which converts the relatively plentiful ribonucleotides (present in cells in low millimolar concentrations) to the much lower concentrations of deoxyribonucleotides (present in cells in low micromolar concentrations) required for DNA replication.

The mechanism of action of the mixture of deoxyribonucleosides in accelerating wound healing is more likely related to regulatory effects of deoxyribonucleotide pools on cell cycling and metabolic regulation than to simply the provision of limiting substrates for DNA synthesis. Cells must necessarily be capable of monitoring availability of deoxyribonucleotides (or their precursor ribonucleotides) as a check point signal in cell cycling, since a deficiency of 1 or more deoxyribonucleotides during DNA replication can lead to either mutations or incomplete replication, both of which can have deleterious consequences in multicellular organisms. This is the basis for the antineoplastic activity of inhibitors of deoxyribonucleotide synthesis such as thymidylate synthase inhibitors (eg, floxuridine [5-fluoro-2'-deoxyuridine]) or antifolates (eg, methotrexate); thymidine starvation is a signal for apoptosis (or active cell suicide) to prevent possible deleterious effects on genomic fidelity.\(^\text{21,22}\)

In hematopoietic cells that undergo apoptosis in response to deprivation of growth factors such as interleukin 3, deoxyribonucleotide imbalances in response to interleukin 3 withdrawal are reported to mediate, not merely accompany, initiation of apoptosis.\(^\text{23,24}\) The implication of this finding is that the converse is probably true; increased availability of particular (or all 4) deoxyribonucleotides may mediate some of the positive stimulatory or mitogenic effects of various growth factors. Augmentation of deoxyribonucleotide pools with exogenous deoxyribonucleosides may either potentiate or bypass elements of the signaling and metabolic events mediating stimulation of cell proliferation (or, perhaps, elaboration of extracellular matrix components) by growth factors.

While all 4 deoxyribonucleosides are required for maximal activity in the rabbit ear ulcer model, the combination of deoxycytidine and deoxyguanosine (or 3-component mixtures containing these 2 compounds) had some degree of efficacy. Nicander and Reichard\(^\text{25}\) and Reichard\(^\text{26,27}\) suggested that deoxycytidine nucleotides in particular have a regulatory role in cell proliferation, since deoxycytidine triphosphate concentrations in proliferating cells rise before initiation of DNA replication, whereas enhanced production of the other 3 deoxyribonucleotides during cell cycling coincides with DNA synthesis.

The enzyme ribonucleotide reductase, which provides the only pathway through which deoxyribonucleotides are synthesized de novo by cells, is directly regulated as a final common control point by a number of positive and negative regulators of cell division.\(^\text{28}\) In the hematopoietic system, the inhibitory effect of several physiological agents on cell cycling can be overcome by providing exogenous deoxycytidine, thus bypassing the need for induction of ribonucleotide reductase, the activity of which rises and falls during cell cycling, reaching a peak just before and during DNA synthesis.\(^\text{29,30}\)

Schwartz et al\(^\text{31}\) reported that various pharmacological treatments that inhibit DNA synthesis specifically by depleting cellular pools of 1 or more of the precursor deoxyribonucleotides in keratinocytes induced their differentiation. While converse experiments investigating effects of superabundance of DNA precursors on keratinocyte differentiation were not discussed or reported, this finding again points toward an important role of deoxyribonucleotide pools in regulation of cell cycling and differentiation.

Determination of the details of the mechanism of action of the mixture of deoxyribonucleosides in accelerating healing may yield important insights into regulation of tissue repair and regeneration. It is striking that both granulation and epithelialization are enhanced by this agent, and that topical administration of the low-molecular-weight deoxyribonucleotides reproducibly accelerates healing in both normal and ischemic wounds to an extent equivalent to or greater than that reported for recombinant wound healing growth factors, ie, platelet-derived growth factor, insulinlike growth factor 1, insulinlike growth factor binding protein 1, keratinocyte growth factor, fibroblast growth factor, granulocyte-macrophage colony-stimulating factor, or transforming growth factor \(\beta1\) in the rabbit ear ulcer model.\(^\text{32-39}\) No other low-molecular-weight agents, whether endogenous metabolites or synthetic compounds, are known to display wound healing activity of this magnitude.
Deoxyribonucleosides are rapidly degraded by enzymes in plasma, erythrocytes, and the liver, so that systemic exposure to topically applied deoxyribonucleosides will be minimal. Patients have received multiple grams of deoxycytidine and thymidine per day by intravenous infusion (for the purpose of modifying toxicity of antineoplastic antimetabolites) with no adverse effects. Moreover, during maturation of erythrocytes, their enucleation releases approximately 1 gram of DNA per day into the circulation (which is degraded to its constituent deoxyribonucleosides by serum enzymes), further indicating that exogenous deoxyribonucleosides applied to wounds are likely to be extremely safe. Furthermore, we have not seen scarring with multiple wound healing agents that are effective in accelerating healing in rabbits. Notably, platelet-derived growth factor does accelerate healing without scar on follow-up, which parallels the human situation for platelet-derived growth factor. However, we have not seen scarring in human trials. Therefore, in view of the safety, stability, and wound healing activity, these deoxyribonucleoside agents hold great therapeutic promise for improving treatment of chronic dermal ulcers and other varieties of wounds seen commonly in the clinic.

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REFERENCES