ACCELARATED HEALING OF INCISIONAL WOUND INDUCED BY INORGANIC POLYPHOSPHATE

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Abstract: Inorganic polyphosphates [poly(P)s] are linear polymers of orthophosphate, that have been shown to be a phosphate reservoir in prokaryotes. Recently, it has been reported that poly(P) enhanced the proliferation of fibroblasts by stabilizing both fibroblast growth factors (FGF) -1 and 2. Since FGFs are unstable endogenous factors for accelerating tissue regeneration, we examined the effect of poly(P) on wound healing by FGF stabilization *in vivo*. The incision wound model was established on rats in the back. Poly(P)- collagen complex was applied on the wounds everyday throughout the experimental period. In control group, only collagen was treated in the same way. Animals were sacrificed at 1,3,5,7, and 14 days after the surgical treatments, and histological evaluation was performed. In poly(P)-treated group, severe neutrophil infiltration was observed in the subcutaneous tissue at 1 day after the treatment, however, inflammatory cells disappeared rapidly and regeneration of defects started at 3 days after the treatment. Regeneration of the skin was clearly observed at 7 days after the surgical treatment, where mature collagen was replaced to the wounded area. In contrast, wound healing of the skin was totally delayed in control group. The expression of type-I collagen mRNA was also analyzed by *in situ* hybridization. At 3 days after the surgery, an increased expression of type1-collagen mRNA was found in poly(P) treated group, whereas little expression was observed in control group. These results suggest that poly(P) could be an effective material for accelerating wound healing.

Introduction

Inorganic polyphosphates [poly(P)s] is a polymer of orthophosphate (Pi) ranging in sizes of up to tens or hundreds of Pi residues. Poly(P) is a completely safe material as evidenced by its use as a food additive all over the world for more than 50 years.

Poly(P) was found in various species from bacteria, insects, plants to mammals [1]. The roles of poly(P) have been investigated mainly in bacteria and shown to function as a phosphate and energy reservoir, a factor for regulating expressions of stress inducible genes, and a component in competence for DNA entry and transformation and so on [1]. Although poly(P) has also been identified in the nucleus and mitochondria of mammalian cells [2], the physiological roles and function in mammalian cells are still obscure.

It has previously suggested that poly(P) could play an important role in tissue regeneration by promoting the stabilization of fibroblasts growth factor-2 (FGF-2) *in vitro* [3]. Poly(P) also strongly stabilizes a complex of FGF-2 and its cell surface receptor and enhances proliferation of fibroblasts [3]. Since FGF-2 is considered to participate in the active tissue regeneration and accelerate wound healing, we designed a new bio-material using sodium polyphosphate. In this study, we examined the possibility of local application of poly(P) on wound healing *in vivo*.

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Materials and Methods

Animals

Six-week-old male Wistar rats (weight about 150 g) were used in this study. The animals were kept on a 12-hours light/dark cycle, fed a standard pelleted diet, and allowed free access to water. This experiment was approved by the Animal Committee of the Hokkaido University Dental School.

Figure 1: Poly(P)-collagen complex.

Materials

First, poly(P)-collagen complex was prepared. Long chain poly(P) was separated as follows. Twenty-grams of sodium polyphosphate with average chain length of about 33 phosphate residues was dissolved in 200 mL of purified water, and then 32 ml of ethanol was gradually added to the solution. The solution was vigorously agitated and then allowed to stand at room temperature for approximately 30 minutes. Centrifugation (10,000×g, 20 min) was performed to separate the precipitate from the aqueous solution. The aqueous fraction was removed, and the precipitated pellet was washed by 70% ethanol. After the precipitate was freeze-dried, 9.2 g of long chain-poly(P) was obtained with average chain length of about 60 phosphate residues.

Using this long chain-poly(P), poly(P)-collagen complex was prepared as follows. Five-grams of long-chain poly(P) was dissolved in 50 mL of sterile distilled water. A chicken-derived atelocollagen (14.3 g) was added to poly(P) solution, thereby generating a gel precipitate (Fig. 1). The resulting precipitate was dispersed by ultrasonication, resulting in gel solution containing poly(P)-collagen complex. This gel solution was composed by 0.1M poly(P) and 1.64 mg/mL of collagen.

Surgical procedure

Rats were anesthetized by ether inhalation. The back of rat was clipped first, then 20 mm-long full-thickness incisional wound was made down to the fascia. To create spindle-shaped wound, the both center edge of the wound was sutured with 6-0 thread in 5mm-width (Fig. 2). In the experimental group, poly(P)-collagen gel solution was applied to the wound. Poly(P)-collagen was given every day through the experimental periods. Collagen gel without poly(P) was applied to the control group by the same way. Rats were sacrificed at 1, 3, 5, 7 and 14 days after the operation.

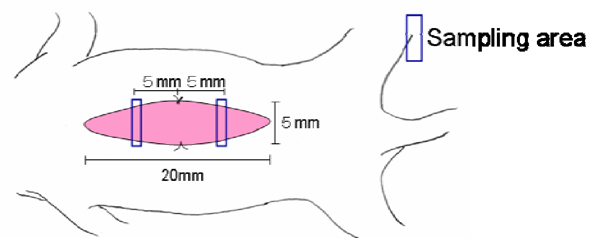

Figure 2: Incision model for wound healing.

Histopathological analysis

After skin samples were taken, they were fixed in 10% phosphate-buffered formaldehyde, and then embedded in paraffin. Sections (4 μm) were deparaffinized and stained with hematoxylin / eosin (HE) and AZAN according to standard procedures.

In situ hybridization

In order to evaluate the localization of cells expressing type-I collagen mRNA in response to type-I collagen during the wound healing process, non-isotopic in situ hybridisation for type-I collagen was performed. Total RNA was isolated from rat skin tissue. Reverse transcription was carried out using ReverTraAce (TOYOBO, Japan), then mRNA was converted into single stranded cDNA using an oligo d(T) primer. The partial sequence of type-I collagen

(nucleotide number 4884-5250) was amplified by PCR using primers FW: 5'-gagggggtttctgtgtcct-3' and RV: 5'-cgaggtagtctttcagcaacaca-3'. The fragments amplified by PCR were inserted into the vector pCR-II TOPO (Invitrogen) to construct pCRII-R type-I collagen.

Skin samples were fixed by 10% paraformaldehyde and embedded in paraffin, and then they were cut in 5-μm thickness and sectioned for *in situ* hybridization analyses. After linearization of pCRII-R type-I collagen, sense and antisense digoxigenin-labeled RNA probes were generated using an RNA labeling kit (Roche Applied Science). Hybridization signals were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody [4].

Results

Histopathology of wound healing

Histological observations of the wound healing were performed as described in Materials and Methods. At 1 day after the surgery, wounded area was filled with blood clots and infiltration of inflammatory cells, which mainly included neutrophils, were observed in both groups. However, inflammatory cells existed in widespread subcutaneous tissue in control group. On the other hand, they were restricted in wound surface area in the poly(P)-treated (experimental) group. At 3 day after the surgery, infiltration of inflammatory cells was still observed remarkably in the control group. However, in the experimental group, inflammatory cells almost disappeared and there could be seen neoangiogenesis, mature fibroblasts, newly formed connective tissues, lymphocytes, and macrophages. Furthermore, the extended epidermis was seen in the both edges of wound surface. At 7 days after the surgery, inflammatory cells including macrophages or lymphocytes still remained and immature fibroblast appeared in control group. In contrast, inflammatory cells were drastically disappeared and there found mature collagen matrix formation in wound area in the experimental group. These results indicated that dermal regeneration was dramatically accelerated by poly(P). At 14 days after the surgery, although wound surface were completely covered with epidermis both in the control and the experimental groups, larger amount of mature collagen fibers were observed only in the experimental group.

In situ hybridization

In the control group, the expression of type-I collagen mRNA was not seen on day 3 in wound area. In contrast, the expression of type-I collagen mRNA was found in both edges of wound area, particularly in cytoplasm of numerous fibroblasts, in the experimental group. At 7 day after the surgery, almost the same levels of expression of type-I collagen mRNA were detected both in the control and the experimental group. At 14 days after the surgery, the expression of type-I collagen mRNA was still observed in the control group, whereas the expression of type-I collagen mRNA in the experimental group was almost disappeared.

Discussion

Poly(P) is a safe and economical material that have been used as food additives all over the world. However, there has been no report that describes pharmacological activity of poly(P). In this study, we first demonstrated the effect of poly(P) on acceleration of wound healing using an animal model. The histopathological analysis clearly showed the rapid infiltration and disappearance of inflammatory cells was observed in the experimental group at early stage of wound healing (within 3 days after the surgery), whereas continuous inflammation was observed until 7 day after the surgery in the control group. This suggests that poly(P) may regulate inflammation efficiency properly and create the best condition for rapid tissue regeneration. This poly(P) effect could be resulted not only in the stabilizations of FGFs and FGF-FGF receptor complexes but also in the stabilization of other proteins such as cytokines involving in regulations of inflammation. The accelerated expression of type-I collagen mRNA, followed by the rapid maturation of collagen fibers could be caused by enhancement of fibroblast proliferation by FGFs stabilized by poly(P).

Conclusion

Poly(P) could be an effective material for accelerating wound healing.

References

[1] KULAEV, I. S., VAGABOV, V. M., and KULAKOVSKAYA, T. V. (2004): 'The biochemistry of inorganic polyphosphates', (J. Wiley and Sons, West Sussex, England), pp. 3-118

[2] SCHRÖDER, H. C., LORENZ, B., KURZ, L., and MÜLLER, W. E. G. (1999): 'Inorganic polyphosphate in Eukaryotes: enzymes, metabolism and function', in SCHRODER, H. C., and MULLER, W. E. G. (Ed): 'Progress in molecular and subcellular biology', Vol. 23 (Springer-Verlag, Berlin), pp 45-81

[3] SHIBA, T., NISHIMURA, D., KAWAZOE, Y., ONODERA, Y., TSUTSUMI, K., NAKAMURA, R. (2003): 'Modulation of mitogenic activity of fibroblast growth factors by inorganic polyphosphate', *J. Biol. Chem.*, 278, pp. 26788-26792

[4] SAMBROOK, J., FRITSCH, E.F., MANIATIS, T. (1989): 'Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory', (Cold Spring Harbor)

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