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Matrix metalloproteinase (MMP) 9 induced in skin and subcutaneous tissue by implanted chitin in rats

T. Nakade[a,*], H. Yokota[b], H. Taniyama[c], Y. Hori[a], N. Agata[a], T. Ikeda[a], H. Furusaki[a], Y. Yamada[a], Y. Uchida[a], A. Yuasa[b], M. Yamaguchi[d], K. Otomo[a]

[a]*Laboratory of Veterinary Surgery 2, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan*
[b]*Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan*
[c]*Laboratory of Veterinary Pathology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan*
[d]*Muscle Biology Research Lab, Department of Veterinary Bioscience, Ohio State University, Columbus, OH 43210, USA*

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Abstract

Chitin was found to induce matrix metalloproteinases (MMPs) activity in rat skin and subcutaneous tissue. Sponge type chitin (22.5 mg) was implanted in subcutaneous tissue of 8-week-old rats by skin incision. MMPs activity was more pronounced in the chitin-treated group than only incision group until on day 2.5 postoperatively. Gelatin zymography revealed that the induced MMPs had a molecular mass of 92 and 82 kDa corresponding to MMP-9 and pro MMP-9, respectively. We here discuss the mechanism of MMP induction by chitin. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chitin; Matrix metalloproteinase; Induction; Zymography; Rat; Skin and subcutaneous tissue

1. Introduction

Because chitin and chitosan have such dramatic effects on wound healing, they have been used in human and veterinary medicine (Bartone & Adickes, 1988; Minami et al., 1992, 1993; Kifune, 1992; Nakade et al., 1996; Okamoto et al., 1993b; Okamoto, Shibazaki, Minami, Matsuhashi, Tanioka & Shigemasa, 1995). The mechanism underlying those dramatic effects is thought to activate macrophage and polynuclear leukocyte infiltration. Matrix-degrading proteases, including matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), are involved in the modulation of the extracellular matrix. MMPs and TIMPs are associated with inflammatory and degenerative processes. For wounds to heal, it is necessary to destroy the extracellular matrix, which consists of the base membrane and interstitial tissue. Sixteen molecular types of MMP have been identified (Nagase, 1997). We investigate the activity of MMPs in the wound healing process, using implanted chitin in rats.

2. Materials and methods

2.1. Preparation of chitin

Chitin sponge (Polymeric *N*-acetyl-D-glucosamine) from squid pen (Sunfive Inc., Japan) with $\leqq$ 10% deacetylation was used.

2.2. Animals

Forty-two 8-week-old male Wistar rats weighing 270–290 g were used. They were divided equally into two groups, with and without implanted chitin. Under general anesthesia, the dorsum back was opened and the chitin (22.5 mg) was implanted or not, and sutured. Each group was divided into seven sub groups based on the days after chitin implantation. Three rats each were euthanasied on day 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 7.0 after being implanted, and samples were collected.

2.3. Preparation of enzyme solution

Rat skin and subcutaneous tissue were sliced into 3 × 3 cm^2 strips. The tissues were cut in pieces and homogenized with a 50 mM Tris phosphate buffer (pH 7.5) containing 0.01 M $CaCl_2$ and 0.25% Triton-X. The homogenates were centrifuged at 8,000 rpm for 15 min at 4°C and the

* Corresponding author. Fax: + 81-11-387-5890.
E-mail address: tnakade@rakuno.ac.jp (T. Nakade)

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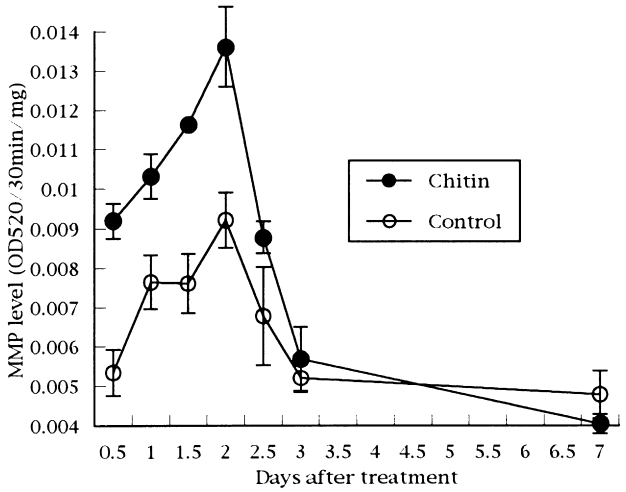

Fig. 1. Change of MMPs activity.

supernatants aspirated. The supernatants were further centrifuged 50,000 rpm for 15 min at 4°C and collected for analysis. The protein concentration was measured by the Lowry method.

2.4. MMPs assay

MMP was routinely assayed using Azocoll as a substrate. The enzyme solution was adjusted to a volume of 1 ml with assay buffer containing 0.05 M Tris–HCl, pH 7.5, 0.2 M NaCl, 0.01 M $CaCl_2$, 0.02% NaN_3, and 0.05% Briji-35. Azocoll (1 mg) was added to a 0.25 ml buffer with a wide-mouthed pipette. Assay tubes were capped and shaken in a horizontal position at 37°C. Aminophenylmercuric acetate (0.5 mM) was used to activate latent enzyme and blanks were prepared with 1 mM 10-phenanthroline. Azocoll was omitted from the assay buffer as a blank solution. Enzyme reaction was stopped by filtration with glass wool to remove the Azocoll substrate. Then, the absorbancy of digested Azocoll in the filtrates was determined spectrophotometrically at 520 nm.

2.5. Zymography

Gelatin substrate gels were prepared by incorporating gelatin (1 mg/ml) into 10% polyacrylamide gels containing 0.1% SDS. Equal amounts of enzyme solution (45 μg protein) were loaded onto the gels. After electrophoresis under non-reducing conditions, the gels were washed for 30 min at room temperature in 2.5% Triton X-100 and subsequently incubated twice for 30 min at room temperature in a Ca^{2+} assay buffer consisting of 1 mM $CaCl_2$ in 0.1 M Tris–HCl (pH 7.5). They were then incubated for 12 h at 37°C in the Ca^{2+} assay buffer. Gels were stained with Coomassie brilliant blue for 60 min and destained in 10% acetic acid 25% ethanol. A clear zone of lysis against a blue background indicated MMP activity.

3. Results

3.1. Activity of MMPs

The levels of MMP using chitin were accelerated 0.5, 1, 1.5 and 2 days postimplantation, and then decreased at 2.5, 3 and 7 days. In the controls without chitin, they were also increased 0.5, 1, 1.5 and 2 days postimplantation and then decreased at 2.5, 3 and 7 days. The activities of MMPs with chitin were markedly higher than in the control at 0.5, 1, 1.5, 2 and 2.5 days postimplantation (Fig. 1).

3.2. Zymography

Gelatin zymography demonstrated that MMP9 (92 kDa) and pro MMP9 (82 kDa) when using chitin were more highly expressed than control on 0.5, 1 and 1.5 days postimplantation. They were more strongly expressed than control on day 2 (Fig. 2). MMP9 (92 kDa) was slightly expressed on day 2.5. There is no difference in MMP9 (92 kDa) and pro MMP9 (82 kDa) on days 3 and 7 postimplantation.

4. Discussion

Chitin and chitosan have medically utilizable biological activities, including those on polymorphonuclear cells and macrophage infiltration, fibroblast, endothelium, newly formed capillary proliferation, acceleration of granulating tissue formation, and enhancement of wound healing (Kifune, 1992; Minami et al., 1992, 1993; Nakade et al., 1996; Okamoto et al., 1993b, 1995; Kosaka, Kaneko, Nakada, Matsuura & Tanaka 1996). Three stages of wound healing were inflammation, granulation and cicatrization. Polymorphonuclear cells and macrophage produced MMP9. The macrophage, fibroblast and endothelium produced MMP2 (Okada, 1995). The level of MMP on day 0.5 postimplantation with chitin was higher than in the control. Polymorphonuclear cells and macrophages infiltrated the injured part before the control. This finding was considered to be caused by chitin. And then polymorphonuclear cells and macrophages produced MMP9. This result is based on previous report (Okamoto et al., 1993a; Usami et al., 1994). The MMP level increased on day 2 in control, due to the polymorphonuclear cell and macrophages infiltration in the normal process of wound healing. This phenomenon was enhanced by chitin. More polymorphonuclear cells and macrophages concentrated around chitin on day 2 postimplantation were than in the control group. Thus, the level of MMP on day 2 postimplantation with chitin was higher than in the control and highest among the chitin groups. In fact, macrography revealed good granulation on day 2 postimplantation with chitin. If we implanted chitin once again on day 1 postimplantation of the experiment, we could maintain the high MMP concentration after day 2.

Gelatin zymography expressed the activation of 92 and 82 kDa molecular weights in the chitin group, i.e. MMP9 and pro MMP9, respectively. They were more strongly expressed than in the control on day 2 postimplantation. Highly-activated MMP9 and pro MMP9 were derived from polymorphonuclear cells and macrophages. We confirmed that polymorphonuclear cells and macrophages showed immunopositivity against MMP-9 antibody. Furthermore, macrophages released platelet-derived growth factor, fibroblast growth factor, and transforming growth factors (TGF)- α and TGF-β. The polymorphonuclear cells secreted IL-1 and accelerated the proliferation of fibroblast and endothelium. The high concentration of MMP induced regeneration of the extracellular matrix. These results indicate that chitin accelerated the effects on wound healing. We conclude that chitin triggers the induction of MMP-9.

References

Bartone, F. F., & Adickes, E. D. (1988). Chitosan: effects on wound healing in urogenital tissue: preliminary report. *Journal of Urology*, *140*, 1134–1137.

Kifune, K. (1992). Clinical application of chitin artificial skin (Beschitin W). In C. J. Brine & P. A. Sandford & J. P. Zikakis (Eds.), *Advances in Chitin and Chitosan*, (pp. 9–15). London and New York: Elsevier.

Kosaka, T., Kaneko, Y., Nakada, Y., Matsuura, M., & Tanaka, S. (1996). Effect of chitosan implantation on activation of canine macrophages and polymorphonuclear cells after surgical stress. *Journal of Veterinary Medical Sciences*, *58*, 963–967.

Minami, S., Okamoto, Y., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, T., & Tokura, S. (1992). Application of chitin and chitosan in large animal practice. In C. J. Brine & P. A. Sandford & J. P. Zikakis (Eds.), *Advances in Chitin and Chitosan*, (pp. 61–69). London and New York: Elsevier.

Minami, S., Okamoto, Y., Tanaka, S., Sashiwa, H., Saimoto, H., Matsuhashi, A., & Shigemasa, Y. (1993). Effects of chitosan on wound healing. In M. Yalpani (Ed.), (pp. 141–152). *Carbohydrates and Carbohydrate Polymers*, 1. ATL Press, chap. 13.

Nagase, H. (1997). Activation mechanisms of matrix metalloproteinases. *Biology and Chemistry*, *378*, 151–160.

Nakade, T., Uchida, Y., Otomo, K., Taniyama, H., Okamoto, Y., Matsuhashi, A., & Minami, S. (1996). Accelerated effect of a natural polysaccharide β-chitin, on wound healing in dogs. *Journal of Japanese Veterinary Medical Association*, *49*, 249–252.

Okada, Y. (1995). Proteinase and tissue destroy. In T. Sakakura (Ed.), *Extracellular*, (pp. 88–99). Tokyo: Youdansha.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y., & Tokura, S. (1993). Polymeric *N*-acetyl-D-glucosamine (chitin) induces histionic activation in dogs. *Journal of Veterinary Medical Sciences*, *55*, 739–742.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., Tanigawa, T., Tanaka, Y., & Tokura, S. (1993). Application of polymeric *N*-acetyl-D-glucosamine (chitin) to veterinary practice. *Journal of Veterinary Medical Sciences*, *55*, 743–747.

Okamoto, Y., Shibazaki, K., Minami, S., Matsuhashi, A., Tanioka, S., & Shigemasa, Y. (1995). Evaluation of chitin and chitosan on open wound healing in dogs. *Journal of Veterinary Medical Sciences*, *57*, 851–854.

Usami, Y., Okamoto, Y., Minami, S., Matsuhashi, A., Kumazawa, N., Tanioka, S., & Shigemasa, Y. (1994). Migration of canine neutrophils to chitin and chitosan. *Journal of Veterinary Medical Sciences*, *56*, 1215–1216.