BIODEGRADABILITY OF ‘MINIPELLET,’ A NEW DRUG FORMULATION USING ATELOCOLLAGEN AS A DRUG CARRIER MATERIAL, IN THE RHESUS MONKEY

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ABSTRACT

‘Minipellet’ is a new drug formulation using atelocollagen as a drug carrier material. We studied the biodegradability of the minipellet formulation histopathologically and biochemically. The minipellets administered to rhesus monkeys for 26 consecutive weeks were examined under the light microscope and, also, by transmission electron microscopy. Furthermore, collagenase activity, tissue inhibitor of metalloproteinase (TIMP-1), and collagen levels were determined. The results suggested that the atelocollagen in the implanted minipellet had been metabolized, chiefly by fibroblasts and macrophages that had invaded into the pellet, in the same way as occurs in tissue remodeling and had been replaced by the host collagen. Analysis of the mechanism of degradation involved showed that whereas no collagenase activity was detected in the pellet or in the tissue adjacent to the pellet, the phagocytosis of atelocollagen by the invading cells was observed, suggesting that the dominant route of pellet decomposition is intracellular degradation.

Many studies have been reported on the application of biocompatible materials, particularly biodegradable polymers, as drug carriers for the controlled release of various drugs. Polylactate and polylactate-polyglycolate are typical carriers (11). However, many of these studies involve the use of organic solvents, heating and/or application of pressure, owing to the physical and chemical properties inherent in the polymers; and it is generally acknowledged that these carriers are unsuited for the preparations of protein drugs.

Recently in collaboration with Koken Co., Ltd., we developed a ‘minipellet’ formulation using a highly purified bovine type I atelocollagen as a drug carrier material (7, 8). Fig. 1 shows an exterior view of the minipellet. The minipellet is prepared by subjecting a highly concentrated solution of atelocollagen to gradual drying. Since the manufacturing conditions for the minipellet are thus mild, the process is considered suited to the manufacture of controlled release formulations of protein drugs, and has so far been applied to interferon-α, interleukin 2, and NGF among others (9, 22). Particularly a minipellet of interferon-α (SM-10500) is already in the clinical evaluation stage in Japan (23).

Atelocollagen is extracted from bovine calf skin by pepsin digestion. In this process the non-helical region of collagen, or telopeptides, which are major antigenic site, are removed (Fig. 2). Therefore, atelocollagen has only a negligible antigenicity, and is broadly used clinically for augmentation of the soft tissue contour as well as for other purposes, with a half of million people having undergone intradermal injections of this material. Its

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biodegradability has been well investigated morphologically (3, 4, 18). However, little research has been done into the mechanism of degradation of the implanted collagen. Moreover, the biodegradability of the non-fibrillar atelocollagen compacted to a hard consistency, as it is in the case with the minipellet, is little understood.

In the view of this situation, we administered the minipellet subcutaneously to rhesus monkeys for 26 consecutive weeks and performed histopathological and biochemical evaluations concerning its biodegradability. After sacrifice of the rhesus monkey, some samples of the subcutaneous tissue and the pellet were stained with hematoxylin and eosin (H and E) and examined light-microscopically, while other samples were examined by transmission electron microscopy. Furthermore, collagenase, which is an enzyme playing a key role in the biodegradation of type-I collagen, and TIMP-1 (tissue inhibitor of metalloproteinases-1), which is a common intrinsic inhibitor of collagenase and other matrix metalloproteinases, were extracted from yet other samples for biochemical assessment.

MATERIALS AND METHODS
Materials and Reagents
Human serum albumin (HSA; Buminate 25%, Sumitomo Pharmaceutical, Osaka, Japan) and atelocollagen derived from bovine skin collagen (Koken, Tokyo, Japan) were used for the preparation of the minipellet. Hydroxyproline (Sigma) was used for the assay of collagen. All the other reagents were of analytical grade (Nacalai Tesque, Japan).

Preparation of the Minipellet
The method for preparation of the minipellet was reported previously (7, 8). In brief, the HSA solution was mixed with a solution of atelocollagen, and the mixture was then lyophilized. The spongy lyophilized material was next swollen by addition of a small amount of distilled water to give a highly concentrated collagen solution. This mixture was extruded through a nozzle to form a rod. The rod was air-dried gradually and thereafter cut to a length of 10 mm to provide transparent cylindrical pellets with a tinge of yellow. The concentration of HSA in each pellet was controlled at 30% (w/w).

Experimental Animals and Administration of the Minipellet
Experiment A Six rhesus monkeys (Kasho, Japan), 40–44 months old, were used in this experiment. At the back of each animal, one minipellet was subcutaneously implanted on a weekly basis for 26 consecutive weeks. Twenty-seven weeks after the beginning of administration, the animals were sacrificed; and the back skin of each animal was taken out, from which, 26 pellets and the subcutaneous tissue around the pellets were cut out after gross observation. These samples were fixed in neurally buffered 10% formalin; some samples (obtained at 1, 3, 11 and 20 weeks after administration) were prefixed in 2.5% glutaraldehyde and postfixed in 2% osmium tetroxide for electron microscopic observation.

Experiment B One male rhesus monkey, 79 months old, was used in this experiment. The animal received one minipelle: subcutaneously into the back weekly for 24 weeks and two pellets weekly two additional weeks. Twenty-seven weeks
after the beginning of the experiment, the animal was sacrificed; and the pellets and subcutaneous tissue (skin) around them were taken out. Each sample was separated into pellet and subcutaneous tissue very close to the pellet, and the wet weight of the pellet was determined. The respective samples were used for the assay of collagenase activity or the determination of the amount of collagen by the methods described below.

**Histological Observation**

The pellets were fixed in neutrally buffered 10% formalin. Then they were dehydrated with ethanol and embedded in paraffin. The samples were thin-sectioned at about 6 μm, and these sections were stained with H and E for light-microscopic observation.

**Electron Microscopic Observation**

Pellets fixed with 2.5% glutaraldehyde were post-fixed with 2% osmium tetroxide and then embedded in epoxy resin. Ultrathin sections were prepared, double-stained with uranium acetate and lead citrate, and examined by transmission electron microscopy (JEM-1200 EX Model II, JEOL, Japan).

**Assay of Collagenase and TIMP-1**

By use of a homogenizer (Wheaton, U.S.A.), collagenase and TIMP-1 in the tissue close to the pellet were extracted in approximately 30 vol of 30 mM Tris HCl buffer (pH 7.6) containing 4M urea. Collagenase and TIMP-1 in the pellet were extracted with the same solution supplemented with ammonium sulfate to 30% saturation to prevent dissolution of atelocollagen in the pellet. These extracts were respectively dialyzed against 30 mM Tris-HCl buffer (pH 7.8) containing 5 mM CaCl₂ and 0.2 M NaCl for two days. Collagenase activity was determined by the method of Terato et al. (19). An ELISA kit (Fuji Chemical) was used for determining TIMP-1 (13).

**Quantitative Determination of Collagen in the Minipellet**

The removed pellet was dissolved in 40 ml of 0.5% HSA (pH 2.0), the pH of which had been adjusted with HCl, with gentle shaking at 4°C for two days. The insoluble fraction was separated by centrifugation at about 10,000 × g and washed twice with
diluted HCl solution (pH 2.0). The soluble fraction, 50 μl, and the precipitate were respectively dried under vacuum and hydrolyzed in 6 N HCl at 110°C for 18 h. The hydrolyzate was evaporated to dryness in a vacuum at 60°C and redissolved in distilled water. The hydroxyproline content of each sample was determined by the method of Bergman and Loxley (2).

RESULTS

Gross Observation of the Subdermal Fate of the Minipellet

The minipellet was swollen and whitened by the tissue fluid soon after implantation but retained its cylindrical structure (data not shown). Subsequently, the pellet tended to lose this cylindrical configuration, became vague in shape and disappeared as the period of implantation was prolonged. Fig. 3 shows the time course of the score representing the change in shape of the pellet in Experiments A and B. The change in wet weight of the pellet in Experiment B is given in Fig. 4. The pellet swelled in the early stage and tended to disappear almost linearly with time.

Cellular Response to the Minipellet

The cellular response to the minipellet was almost the same in each rhesus monkey. Presented in Fig. 4 are typical pictures showing the cross-section of the pellet and subcutaneous tissue stained with H and E. Since the pellet was more eosinophilic and stained in a lighter shade than the surrounding tissue, it could be easily distinguished from the surrounding tissue. This tendency continued until the pellet ceased to be recognized.

Presented in Fig. 5-1 is a photomicrograph showing the stained minipellet one week after administration. The pellet was swollen and had been fragmented by the tissue fluid; thus it had lost its original homogeneous structure. The cross-sectional shape of the pellet was oval. Polymorphocytes, which were identified mainly as fibroblasts but to a lesser extent as macrophages by electron microscopy, had invaded into the pellet from its periphery. Very slight infiltration by inflammatory cells was found around the pellet. Infiltrating cells were histocytes, lymphocytes and plasma cells, and eosinophils were detected to a very slight degree.

Four weeks after administration, inflammatory cells tended to diminish in number. Instead, fibroblasts had invaded into the pellet (Fig. 5-2). The pellet was further finely fragmented, and many fibroblasts were identified in the crevices. Inflammatory cells had disappeared almost completely by 11 weeks after administration. The whole pellet had been invaded by fibroblasts. Capillary neovascularization was found in some pellets. The pellet itself remained eosinophilic and could be clearly differentiated by staining from the surrounding tissue. However, the cross-sectional area of the pellet had definitely decreased (Fig. 5-3).

Nineteen weeks after administration, the above trends were even more remarkable. The pellet was further fragmented and reduced in size. Many
Fig. 5 Light photomicrographs of minipellets administered subcutaneously to a rhesus monkey (H and E stain, scale bar, 200 μm). 1: One week after administration. The pellet appears finely fragmented. Very slight cellular infiltration can be seen in the tissue around the pellet. Evidence for polymorphocyte invasion into the pellet is visible (inlet; ×10). 2: Four weeks after administration. The pellet remains finely fragmented. Infiltration of inflammatory cells has begun to diminish. Even more polymorphocytes have invaded into the pellet from its periphery. 3: Eleven weeks after administration. The pellet has become further finely fragmented. Infiltrated cells into the tissue around the pellet have disappeared. The pellet has been eroded and diminished in size. Invasion of polymorphocytes into the pellet is evident. 4: Nineteen weeks after administration. The pellet is even further finely fragmented and diminished in size. Many polymorphocytes (arrow), mainly fibroblasts, and new capillaries (arrowhead) are noticeable in the pellet (inlet; ×10).

Fibroblasts and new capillaries were found in the pellet (Fig. 5-4).

*Electron Microscopic Observation of Internal Part of the Pellet*

For morphological assessment of the pellet and invading cells, electron microscopic observation was performed. The findings are shown in Fig. 6. Most of the cells invading the pellet were fibroblasts, with macrophage being sporadically observed. Beginning one week after administration, the electron density of the pellet became irregular, and lots of invaded cell parts were found (Fig. 6-1). The pellet collagen lacked the periodicity characteristic of tissue collagen fibers. One week after administration and onwards, invading cells, fibroblasts or macrophages, engulfed the pellet.
Fig. 6  Transmission electron micrographs of minipellets administered subcutaneously to a rhesus monkey (stained with uranium acetate and lead citrate). 1: One week after administration. The pellet is uneven in electron density and lots of cell part were found (arrow). 2: One week after administration. Invading cells, fibroblasts or macrophages, have engulfed the pellet substance, indicating that the cells phagocytized atelocollagen (arrow). 3: Three weeks after administration. Fibroblasts with well-developed granular endoplasmic reticula (arrowhead) are seen in the pellet. 4: Twenty weeks after administration. The cross-section of newly generated host collagen bundles (arrow) can be seen around the fibroblasts invading into the peripheral portion of the pellet.
Fig. 7  The amount of hydroxyproline in the minipellet in Experiment B. The shaded portions of columns indicate the amount of hydroxyproline in the precipitate that did not dissolve in dilute hydrochloric acid (pH 2.0); and the unshaded portions, the amount that was acid-soluble.

Table 1  Time Course of the Amount of TIMP-I in the Pellet and in the Tissue around the Pellet

<table>
<thead>
<tr>
<th>Time after implantation (week)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>13</th>
<th>17</th>
<th>23</th>
</tr>
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<tr>
<td>In the minipellet (× 10^{-3} mg/pellet)</td>
<td>NA</td>
<td>11.0</td>
<td>6.2</td>
<td>8.5</td>
<td>3.4</td>
<td>1.5</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>In the tissue around the minipellet (mg/g wet tissue)</td>
<td>0.08</td>
<td>1.18</td>
<td>0.60</td>
<td>0.31</td>
<td>0.13</td>
<td>0.05</td>
<td>0.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

NA, not assayed; ND, not detected

material with their processes. This indicates that the cells phagocyzed atelocollagen (Fig. 6, 2 and 3). Some invading fibroblasts had well-developed granular endoplasmic reticula (Fig. 6-3). After 3 weeks, collagen fibrils with periodicity were observed around the invading fibroblasts. This collagen was not so well organized as that in the adjacent subcutaneous tissue, suggesting that this is newly generated host collagen. This change tended to increase with the period of implantation (Fig. 6-4).

**Time Course of Change in Pellet Collagen Content**

The change in the amount of collagen in the implanted pellet was followed by measurement of hydroxyproline (Hyp) as the indicator. The results are shown in Fig. 7. The total amount of Hyp in the pellet declined almost linearly with time. With the passage of time, the acid-solubility of the pellet changed. Thus, whereas the acid-solubility of the pellet before administration was 100%, the pellet that had been in place for a long period tended to become insoluble.

The host collagen molecules that fibroblasts secreted would have telopeptides. Therefore, they would have a tendency to be cross-linked, and as a result, the host collagen fibers would be insoluble as opposed to the atelocollagen. Taken together with the electron microscopic findings the results suggest that, within the pellet, the atelocollagen was gradually replaced by the host collagen.

**Time Courses of Collagenase Activity and TIMP-I Content in the Minipellet and Surrounding Tissue**

It is known that collagenase plays a dominant role in the extracellular degradation of type I collagen. Therefore, we examined collagenase activity both in the pellet and in the surrounding tissue. How-
ever, neither active nor latent collagenase activity was detected at any time after implantation. The level of TIMP-1, which is associated with the modulation of tissue matrix metalloproteinase activity, was also determined (Table 1). Although elevation of the TIMP-1 level in the surrounding tissue was found in the early stage after implantation, the level then declined and ultimately returned to the pre-implantation tissue level. TIMP-1 was also detected in the pellet, but its level was very low.

DISCUSSION

Since 1869 when the cat gut was first utilized as a surgical suture, collagen has been used as a biocompatible material in a broad spectrum of applications, typically a therapeutic material for wounds and a prosthetic skin (3–5). For some of these applications, native collagen was modified by cross-linking with chromic acid or glutaraldehyde in an attempt to prolong its residence time for improved bioavailability. In most instances collagen administered in vivo was eliminated (14, 15).

In the present study, the biodegradability of the minipellet formulation using bovine type-I atelocollagen as a drug carrier material was investigated in the rhesus monkey. As a result, the minipellet was found to disappear like the conventional biocompatible materials. This result suggests that the physical characteristics (shape, hardness, etc.) of the minipellet do not interfere with the biodegradability of atelocollagen itself and that atelocollagen is thus a useful drug carrier material.

As verified histopathologically, the host’s response for disposal of the minipellet was a very mild one, chiefly involving fibroblasts and macrophages. It was also an important finding that the infiltration of inflammatory cells was very slight and faded away in the early stage. It is known that administration of a solid matter may occasionally elicit a foreign-body reaction (16). Furthermore, it is also known that in the case of a material that has antigenic peptides, the host’s immuno-reaction is deployed. In these cases, the infiltration of inflammatory cells around the materials is remarkable and persists for a long period, till the disappearance of the material. Thus, the mode of degradation of the minipellet in this study cannot be equated with that of these cases. The rapidity with which the minipellet swells and loses its hardness in the encounter with the tissue fluid and the low antigenicity of atelocollagen are probably responsible for minimal inflammatory response.

The form of fibroblasts and macrophages invasion into the pellet resembles the tissue remodeling observed in the repair of the skin (6). It is suggested that the essential mechanism of the minipellet degradation is most likely comparable to the metabolic mechanism involved in the tissue remodeling. This assumption could be fortified by the electron microscopic observation and solubility study of collagen, which indicated that fibroblasts not only decomposed atelocollagen but also secreted newly generated collagen. It is known that fibroblasts recognize the three-dimensional structures of tissues and have the function to maintain such structures (24). It is probable that the atelocollagen in the minipellet was recognized by the fibroblasts as a mere unorganized collagen mass. Accordingly, their metabolic activity was stimulated. Tissue repair by fibroblasts was activated and, as a consequence, a portion of atelocollagen in the process of decomposition, was replaced by the host tissue collagen, thus resulting in the ultimate disappearance of the atelocollagen.

It is also known that macrophages are chemotactically sensitive to degradation products of collagen (17) and that through cytokines they control the metabolic activity of fibroblasts in tissue repair (12, 16). It may be conjectured that the metabolic activity of the fibroblasts was also stimulated by factors secreted by these macrophages.

Thus, the exogenous collagen thus introduced would appear to be decomposed by the same degradation mechanism that the host employs for the metabolism of its own collagen. The metabolic mechanism can be roughly divided into two pathways, i.e. extracellular degradation and intracellular degradation (20). In this study the level of collagenase, the key enzyme in the extracellular degradation pathway, was investigated in both the pellet and the tissue surrounding the pellet. However, we could not extract a detectable amount of collagenase with urea from the pellet or the surrounding tissue. The reasons for this may be the binding of collagenase to atelocollagen and inhibition by TIMP-1 that was co-extracted (1). However, the important implication of the result is that there was no sharp elevation of collagenase activity in the implantation site. The validity of this finding is verified by the fact that the tissue level of TIMP-1, which masks collagenase activity, was not appreciably elevated. Usually, collagenase can be detected in the site of a wound or inflammation.
by the method we employed in this study (10). Therefore, the result does not suggest that collagenase did not decompose atelocollagen but rather suggests that collagen remodeling within the pellet is very gentle compared with that in wound healing. Collagenase probably acted in an extremely controlled state and probably in a manner similar to that in the usual metabolic turnover of tissues.

Regarding the intracellular degradation pathway, electron microscopic observation indicates the phagocytosis of atelocollagen by fibroblasts or macrophages. It is clear that macrophages have phagocytic activity, but it is also known that fibroblasts phagocytize unorganized collagen fibrils, especially in the repair of periodontal tissue (21). Probably the three-dimensional structure of unorganized collagen of the pellet activated the phagocytosis by the fibroblasts. This result, though only qualitative, is in contrast to the result observed for collagenase. The present results are not comprehensive enough to state categorically whether the pellet is decomposed extracellularly or intracellularly. Further investigation at the molecular level is needed. However, when the electron microscopic picture is superimposed on the finding that atelocollagen of the milligram order disappeared within a comparatively short time despite the low level of collagenase activity, which was below the detection limit, we are led to conclude that dominant biodegradation of pellet atelocollagen proceeds intracellularly.

In conclusion, atelocollagen, used as a drug carrier, seems to be gently replaced by host collagen through a remodeling process involving mainly fibroblasts and ultimately disappears. As to the mechanism of degradation, intracellular degradation by fibroblasts appeared to be predominant.

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REFERENCES

