Tissue Reaction and Biodegradation of Implanted Hemostatic Collagen Fleece in Rats

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ABSTRACT

The rate of biodegradation of two commercially available hemostatic collagen fibers, i.e., collagen fleece (Novacol) and microfibrillar collagen (Avitene) were studied by biochemical and morphologic methods. Urinary excretion of hydroxyproline as an index of collagen degradation was studied in 10 six–month–old rats after s.c. implantation of 2 g of either material during 15 postimplantation sampling periods for a total of 64 days. It was found that collagen fleece degraded significantly faster than microfibrillar collagen, 50% of implanted collagen fleece being resorbed within 8.5 days as compared to 18.8 days for microfibrillar collagen.

Morphological evaluation of the resorption and corresponding tissue reaction was done in two groups of 6 male Sprague Dawley adult rats surgically implanted subcutaneously, intramuscularly, intrahepatically, intrasplenically and intrarenally with 3 mg samples of collagen fleece or microfibrillar collagen at multiple implantation sites. One animal from each group was sacrificed and samples were obtained at 7, 14, 28 days and at 2 months.

Both types of fibrillar collagen implants induced cellular reaction, with faster ingrowth of cells around and into the collagen fleece implant. With microfibrillar collagen the reaction is delayed and lasts for a longer period of time. Soon after tissue implantation of collagen fleece the implants cannot be identified by the naked eye due to its gelling. Only morphological analysis indicates the magnitude of the resorption of collagen fleece. While collagen fleece was not detected in four week specimens of either tissue, microfibrillar collagen was still present as an encapsulated depot at two months. The differing dynamics of the biodegradation of these two collagen fiber materials are the reflection of structural stability of their molecules.

INTRODUCTION

Collagen is a biodegradable material. Once implanted into tissues of an organism, it will be ultimately degraded and resorbed by the effect of cells such as granulocytes and macrophages. These cells progressively invade the implant and, through their collagenase system, selectively digest collagen. Degradation products are then attacked by various peptidases forming small fragments containing hydroxyproline (Hyp) which are detectable in urine. If the animal is kept on a standard diet and other factors effecting Hyp excretion are kept the same (age, sex, physical activity), the increase in urinary Hyp indicates the magnitude of the digestion and degradation of the implanted collagen. It has been well documented that, besides the geometry of the tissue implant, it is mainly the degree of cross-linking of the collagenous implant (referred to in this paper as structural stability) which controls the rate of biodegradation[1].

The other method of studying the bioresorption of collagenous implants is the direct morphological assessment of the implantation site with documentation of both tissue inflammatory reaction and physical presence of the implant. Tissue reaction can be semiquantitated by scoring some characteristics of the inflammatory tissue reaction, such as cellular infiltrate, vascularization, presence of atypical cells (giant cells, lymphocytes), fibrotic response, presence (absence) of the implant, etc. This approach was used in this study and the methodological details are given below.

MATERIALS AND METHODS

1 Materials

The Hemostatic Collagen Fleece (collagen fleece, Novacol, Bioplex Corp., a subsidiary of Datascope Corp., NJ, USA) used in this study was isolated from bovine Achilles tendons by a combination of chemical and mechanical treatments[2]. The tested material exists in an unwoven web, consisting of pure collagen fibers with an average of 50 μm thickness and 2 to 3 cm length. Microfibrillar Collagen Hemostat (Avitene) was supplied by Avicon Corporation, Fort Worth, TX. It is a whitish powder of microfibers with an average length of 100 Å, manufactured according to Battista and Cruz[3].

2 Animals and treatments

For biochemical determination of collagen degradation by analysis of urinary hydoxyproline (Hyp), a total of 10 young adult Sprague Dawley male rats, 200 ± 20 g body weight were used. Rats were assigned at random to two groups. Each rat was marked and placed in a clean plastic metabolic cage for urine collection. Two consecutive 24 hour urine specimens from each rat were taken and tubes were labeled with rat numbers. Hydroxyproline was determined by Woessner procedure[4] to establish the baseline data for each rat. After implantation a total of 18 samplings of 24 hour urine output was collected for Hyp deter-

mination.

The morphological method used two groups of 6 male Sprague Dawley, 300 g rats surgically implanted subcutaneously, intramuscularly, intrahepatically, intrasplenically and intrarenally with two to five 3 mg samples of collagen fleece (group A) and two to five 3 mg samples of microfibrillar collagen (group B).

Animals were anesthetized with Innovar Vet. 0.3 ml/100 g b.w. (Pittman Moore) and surgical sites, dorsal/pelvic midline, hind leg (thigh) and abdominal (midline) were shaved and prepped with iodine (loprep, Arbrook).

3　Surgical procedures

Surgical implantation of the tested material was done by a sterile procedure. Each rat was anesthetized with Innovar Vet. (Pittman Moore), shaved and prepped with Betadyne surgical scrub. Rats used for the biochemical method had a single 2.0 cm long dorsal incision made through the dermis through which a large subcutaneous pocket was formed using gentle, blunt dissection. Rats 1–5 received exactly 2.0 g each of microfibrillar collagen. Rats 6–10 received exactly 2.0 g each of collagen fleece. Wounds were closed with skin staples, four per wound. Animals were allowed to recover and returned to the urine collection cages. All rats received 12 ml s.c. saline (sterile) to promote urine output.

An additional 12 rats were implanted with 3 mg samples of collagen fleece or microfibrillar collagen into five various tissues under a similar protocol. Implantation was done through a 0.5 cm long dorsal pelvic midline incision through the dermis to the fascia. A five inch Kelly curved hemostat was used to make a small pocket about 1.0 cm from the proximal end of the incision. Four to five 3 mg samples of collagen fleece or microfibrillar collagen were positioned with the subcutaneous pocket. Closure was made with two interrupted square knots of 4-0 monofilament nylon.

Intramuscular deposits were made into the hind right legs. A small pocket between biceps femoris and caudofemoralis muscle was made about 0.3 cm from the proximal end of the incision.

Intrahepatic implants were placed after an abdominal midline incision from the zyphoid extending distally from 4.0 cm into a 4.0 mm deep puncture wound made on the ventral surface of the left lateral lobe. Moderate bleeding ceased immediately following the implants.

Intrasplenic implants were made on the anterior mesenteric surface 1.0 cm from the anterior pole in a puncture depth that did not exceed 3.0 mm.

Intrarenally placed collagen was implanted on the apex of the lower pole of the left kidney in a depth not exceeding 4.0 mm. Abdominal musculature was approximated with running locked 3-0 proline sutures and the skin was closed with simple interrupted 3-0 proline sutures (Ethicon).

4　Hyp determination

Woessner's method[4] for Hyp determination was used for urine hydrolysates. This method eliminates the interference of urine chromogens with Hyp determination. After 9

weeks, all rats were sacrificed by anesthetic overdose.

Collected urine samples in test tubes were overlaid with 0.2 ml toluene and kept refrigerated until analyzed for hydroxyproline determination.

The urine analysis included the following steps:

- Centrifugation at 4°C and 4000 r.p.m. (500×g) for 15 min to remove possible contamination by food.
- Determination of volume of urine.
- Take 1 ml aliquot for acid hydrolysis (6N HCl, 105°C, 18 hrs, screw cap test tubes).
- Decolorization of hydrolyzed urine with activated charcoal (Norit), shake, filter rinse filtrate two times with distilled water.
- Dehydration of the deodorized filtrate.
- Dissolution of the evaporated filtrate in 2 ml of distilled water.

Hydroxyproline assay

The possible interference of urinary chromogens in the Hyp assay was also tested by using an internal standard added to the urine before the hydrolysis. An 85% recovery was established.

5　Histology

Samples of the five tissues implanted with either type of collagen, obtained at 7, 14, 28 days and at 2 months, were placed in 10% alcohol–buffered formalin immediately after excision. Five micron sections from each sample were stained with hematoxylin and eosin and identical sections were stained with Masson's trichrome for collagen.

The following criteria and quantitation were used to evaluate the morphological reaction to either implant in the rat subcutis, spleen, liver, muscle and kidney.

  a) Inflammatory cell infiltrations density: 1–no reaction, 2–minimal, 3–moderate, 4–severe.
  b) Edema in the interstitial tissue: 1–none, 2–minimal, 3–moderate, 4–severe.
  c) Capillary ingrowth and new collagen deposition into the site of the implantation: 1–none, 2–minimal, 3–moderate, 4–severe.
  d) Presence of the implant magnitude of resorption: 1–completely resorbed, no traces left, 2–traces, infiltrated by cells, 3–present with limited cell infiltration, 4–intact deposit, cells only around the collagen.

6　Statistical analysis

Biochemical data (**Table 1**) were treated by two–tail ANOVA analysis of variance test. Semiquantitative scores of morphological evaluations used Duncan's multiple range variability test to test the significance.

RESULTS

1　Biochemical study on collagen resorption

A total of 212 twenty-four-hour urine collections were processed for the determination of

Table 1　Urinary hydroxyproline excretion, combined profiles

Day	Microfibrillar collagen	Collagen fleece	Day	Microfibrillar collagen	Collagen fleece
− 1	726. 2 54. 2	640. 5 41. 9	9	794. 2 77. 0	857. 5 83. 7
0	695. 5 64. 9	614. 0 30. 4	14	1425. 2 85. 1	971. 5 39. 5
1	1013. 2 88. 1	3861. 5 606. 6	21	1171. 5 80. 6	903. 8 68. 9
2	2017. 8 116. 4	6613. 5 419. 7	28	1200. 3 102. 8	784. 5 145. 7
3	1901. 0 381. 3	4172. 0 452. 8	35	1010. 0 69. 7	803. 4 52. 2
4	1556. 5 374. 7	2266. 2 386. 8	42	911. 4 51. 5	716. 2 51. 2
5	1209. 8 198. 8	1520. 2 162. 2	49	685. 0 54. 0	690. 9 39. 9
6	1000. 5 258. 8	991. 0 162. 8	56	719. 6 13. 8	659. 8 52. 0
7	864. 6 166. 4	969. 6 101. 4	63	716. 3 19. 0	686. 7 44. 2

Data are presented in micrograms as $X \pm SD$, based on five analyses for either sample at each time sampling period.　For graphic representation see Figs. 1–3.

urinary hydroxyproline as a measure of collagen degradation.　The results, based on five rats subcutaneously implanted either with 2 g collagen fleece or 2 g microfibrillar collagen are shown in **Table 1** and **Fig. 1**.　Note that in **Fig. 1** the data for collagen fleece degradation-derived urinary Hyp are using different Y–axis scale than that for microfibrillar collagen.　In order to make adequate visual comparison, **Fig. 2** presents the same data as those on **Fig. 1** but uses the same scale dimensions.　This presentation clearly documents that during the first four days after implantation of collagen products, microfibrillar collagen implanted rats excreted a total of 6488. 5 μg Hyp, while under the same time period collagen fleece implanted rats excreted 16913. 2 μg Hyp, thus almost three times more.　The magnitude of this difference cannot be attributed to the surgical trauma, which was comparable for all rats, but should reflect some characteristics of the implanted collagen product.　In this respect, it is interesting to note that in contact with aqueous fluids microfibrillar collagen behaves differently from collagen fleece.　Collagen fleece gels quickly to form a homogenous–colloidal milieu with considerable viscosity and gluing properties.　Microfibrillar collagen implants remain more particulate forming a dispersion and not a gel.　Analysis of the patent on manufacturing of microfibrillar collagen led us to believe that it is formed by a more insoluble core of collagen protein (more stable, cross–linked) than the collagen fleece[3].

　　We tried to substantiate this statement by direct determination of shrinkage temperature (T_s) of either product.　We used the birefringence characteristics of native collagen, being
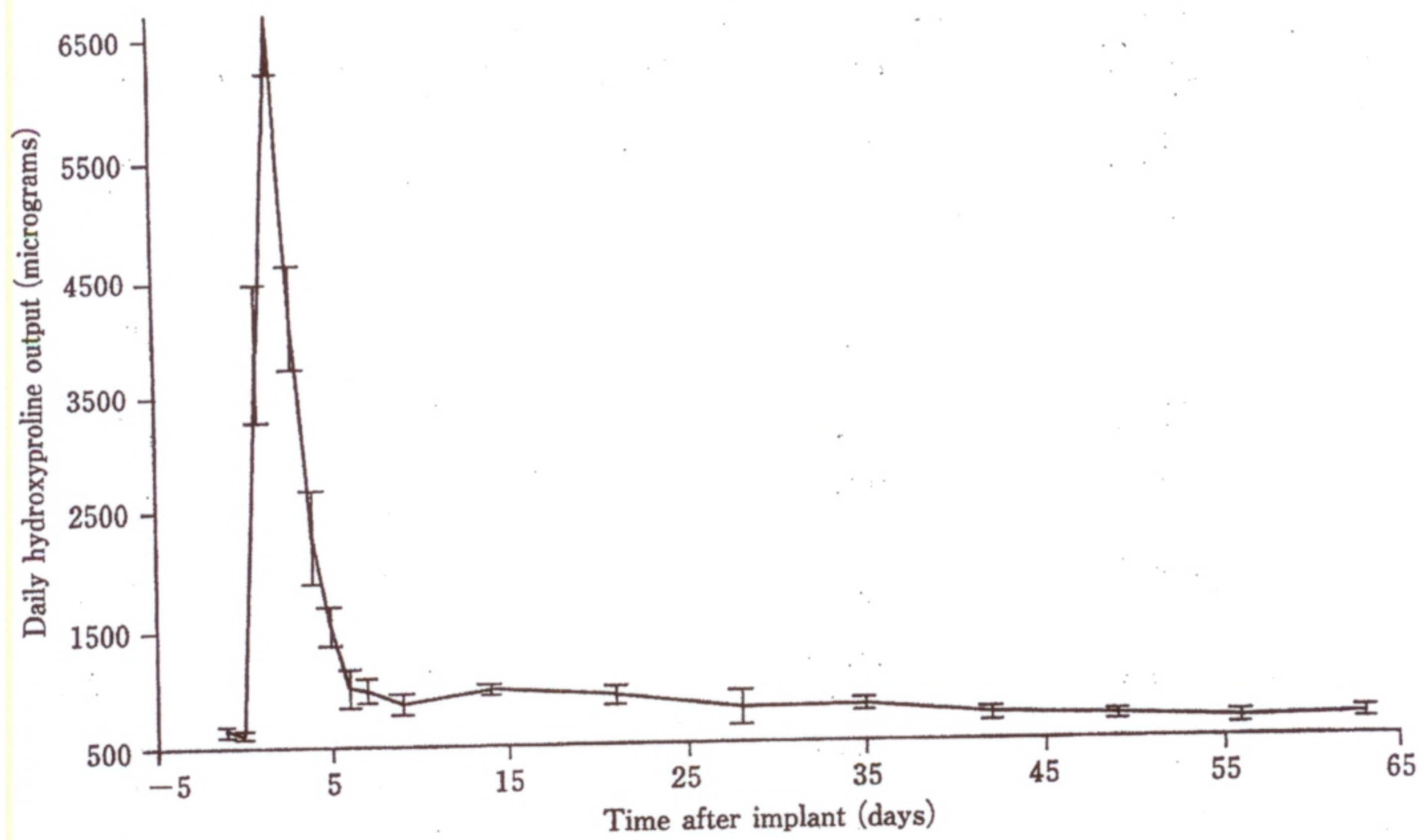
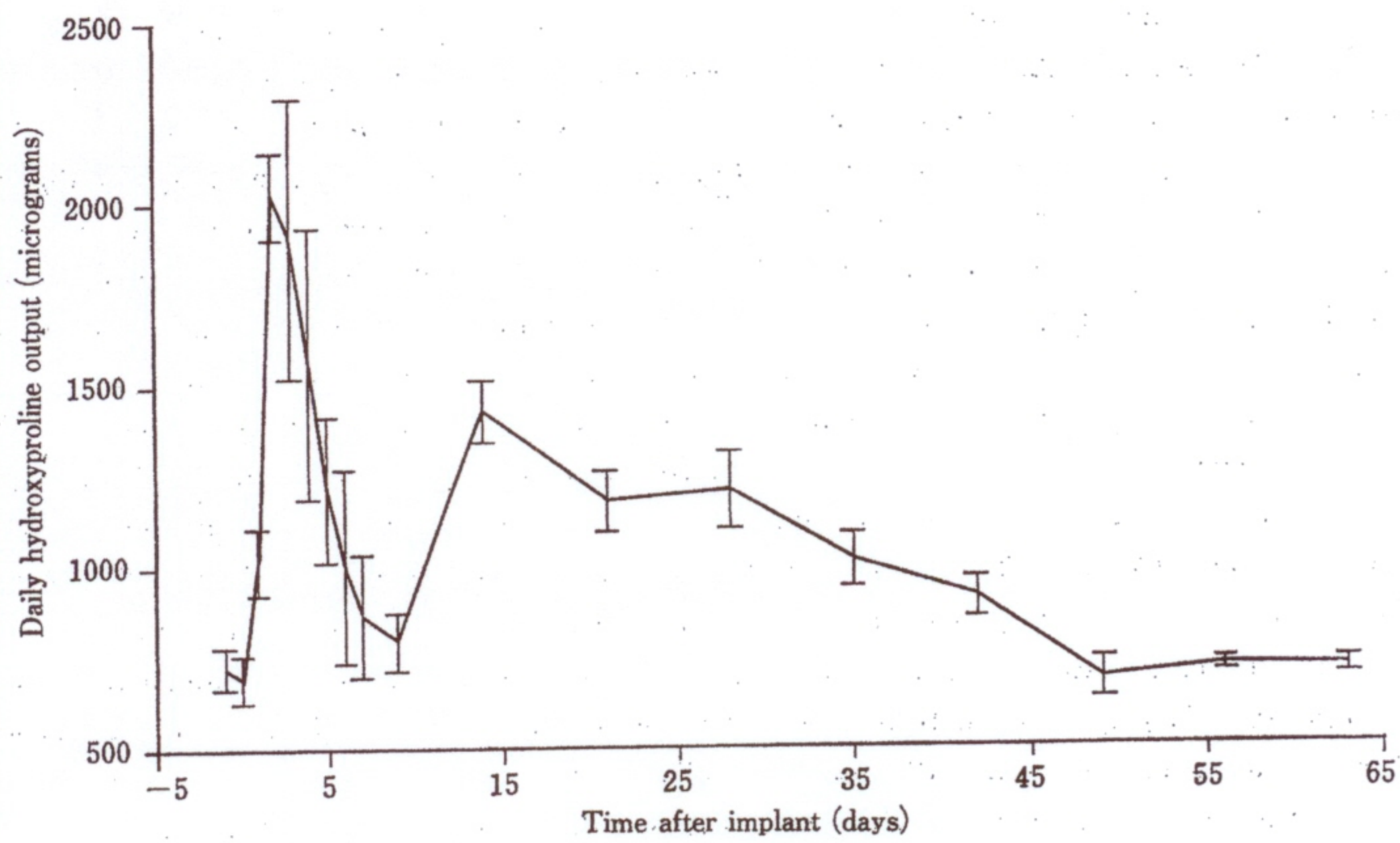**Fig. 1-a** Urinary hydroxyproline excretion, collagen fleece implant

Fig. 1-b Urinary hydroxyproline excretion, microfibrillar collagen implant
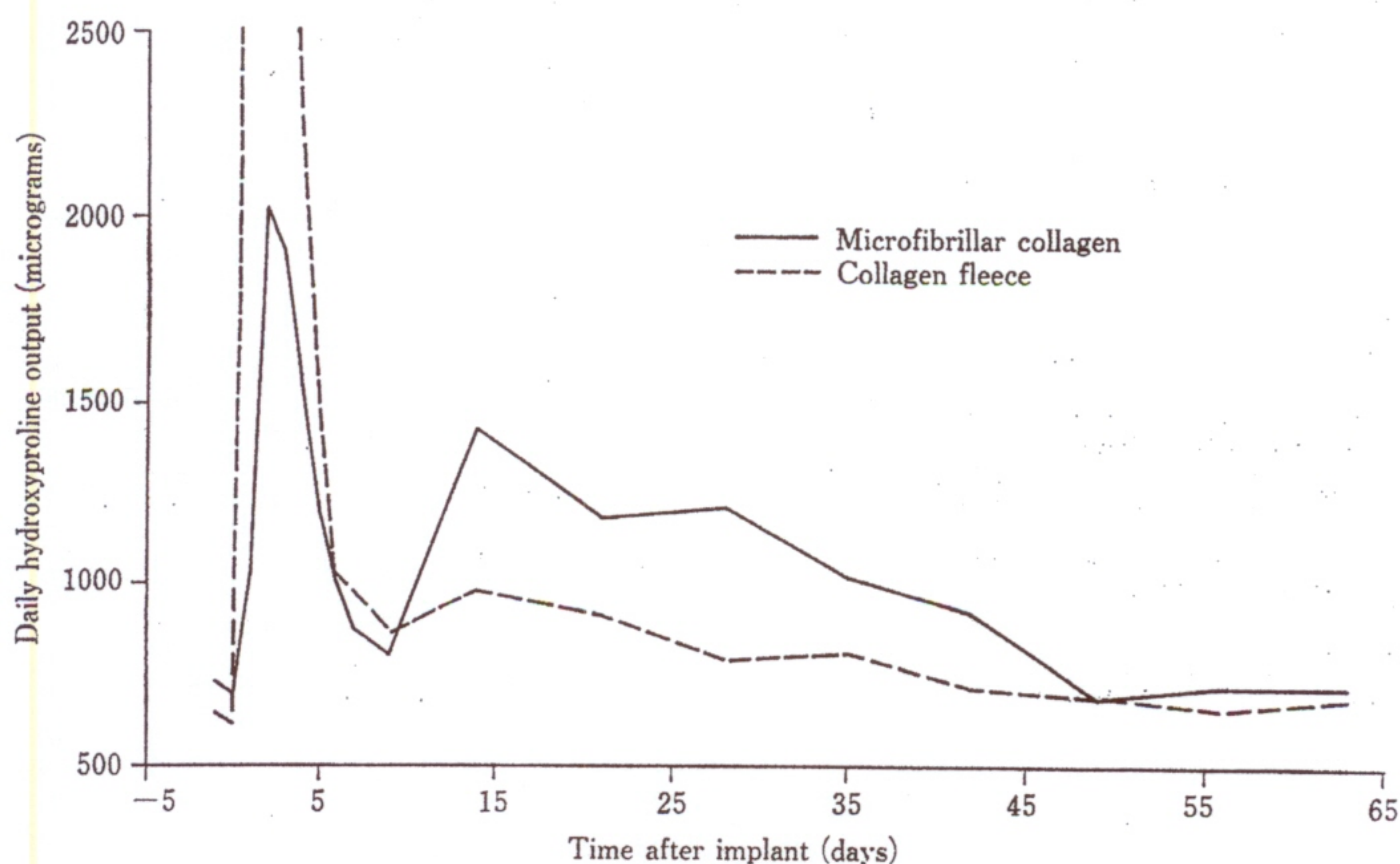
184(3932)

Fig. 2　Urinary hydroxyproline excretion, collagen hemostats implants

anisotropic and then changing into isotropic form at denaturation temperature (T_s). The measurements were done on a polarized microscope fitted with Koeffler heating block, raising temperature of the sample in the capillary in 0.1 N phosphate buffer, pH 7.0 by 2°C/min[5]. We found that there is no sharp peak of T_s in either sample. The range of temperatures indicated rather continuous changes in birefringence to complete isotropicity for: microfibrillar collagen 52–61°C, collagen fleece 46–56°C.

We believe that this finding substantiates our observation on higher structural lability of collagen fleece over microfibrillar collagen. This would explain that within a week after s.c. implantation more than 50% of the implant collagen fleece was degraded while the ramaining part, constituting mostly the insoluble core of collagen fibers was degraded at a much slower rate.

The data presented in **Table 1** were used to obtain further information on the degradation of either form of tested collagen fibers as presented in **Table 2**. In principle, no statistical difference was found between collagen fleece and microfibrillar collagen in the integrated amount of Hyp excreted during 9 weeks of the study. The amount of Hyp excreted derived from degraded collagen and excreted through the urine was 11.5 and 13.5% for collagen fleece and microfibrillar collagen respectively. However, resorption rate of collagen fleece determined as the velocity constant of the curve shown in **Fig. 2**, was significantly higher than that of microfibrillar collagen ($p < 0.002$), thus, the half time of collagen fleece resorption was also significantly shorter than that of microfibrillar collagen ($p < 0.001$).

Using the data on total Hyp excretion through the urine and the rate of implant resorp-

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Table 2　Additional information on biodegradation of collagen hemostats

Parameter	Collagen fleece	Microfibrillar collagen
Implanted amounts	2.0 g	2.0 g
Residual moisture in the implant (in %)	6.6	7.2
Actual amount of collagen implanted	1.87 g	1.86 g
Total Hyp implanted	237 mg	226 mg
Total Hyp excreted during 9 weeks	27.3 mg	30.9 mg
Effectiveness of urinary Hyp excretion of degraded collagen	11.5%	13.5%
Half time of implant resorption	8.5 days***	18.8 days
Rate of resorption of the implant (velocity constant)	0.0819**	0.0369

For details see text.

Asterisks refer to statistically significant difference at $p < 0.01$(**) or $p < 0.001$(***).

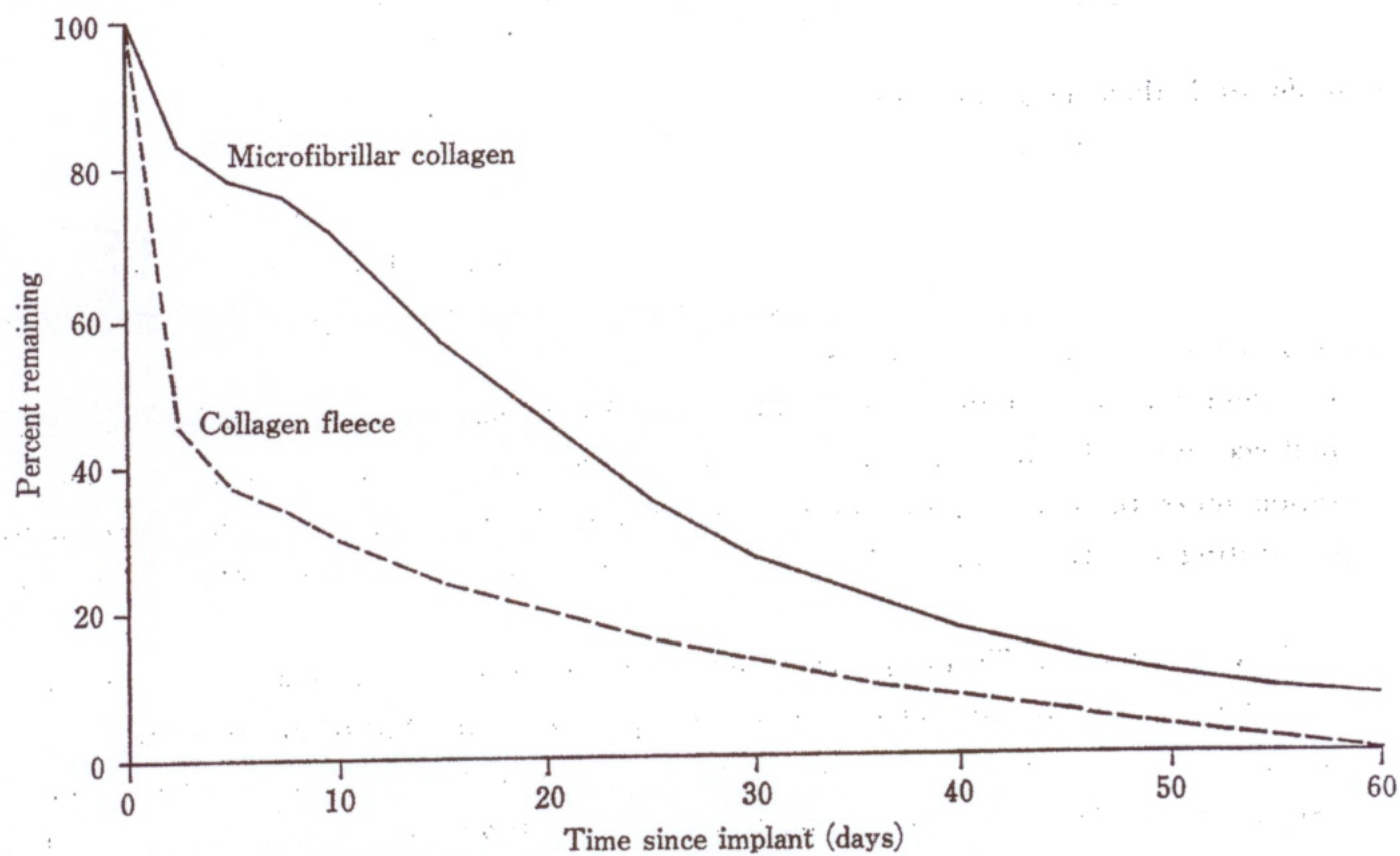
Fig. 3　Collagen resorption curves

Fig. 4 Collagen fleece fibers seven days after subcutaneous implantation (180 × magnification). The implant is swollen, glassy, without noticeable texture. Only few cells penetrate into the implant.

Fig. 5 Microfibrillar collagen implant seven days in the subcutis. Note the individual fibers, invaded by inflammatory cells (180 × magnification).

tion, resorption curves for microfibrillar collagen and collagen fleece were calculated and are shown in **Fig. 3**. In the calculation we subtracted from the analytically determined Hyp content the values of preimplantation hydroxyproline excretion, used factor 7. 46 to convert Hyp values to actual collagen degraded, corrected for only 12% excretion of Hyp by urine. The curves for both collagen fleece and microfibrillar collagen reflect bi-exponential kinetics of collagen degradation, significantly faster for collagen fleece at early postimplantation time as well as later, reaching the baseline level significantly faster than microfibrillar collagen implants.

2 Morphological study of collagen resorption

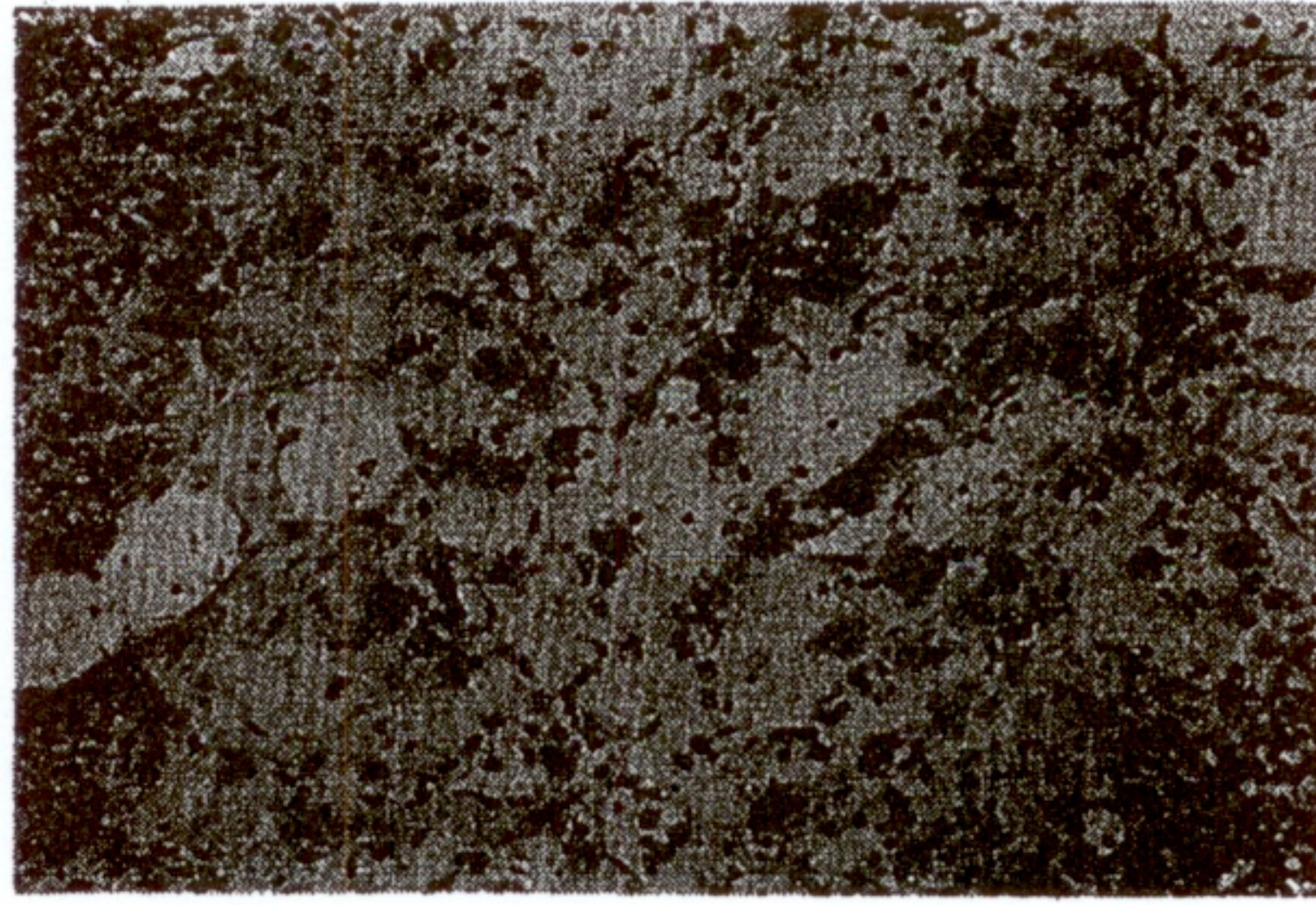Some morphological findings, regardless of the implanted tissue, were typical of the two

Fig. 6　Collagen fleece 14 days after implantation in subcutis is penetrated by inflammatory cells and some vessels (180× magnification).
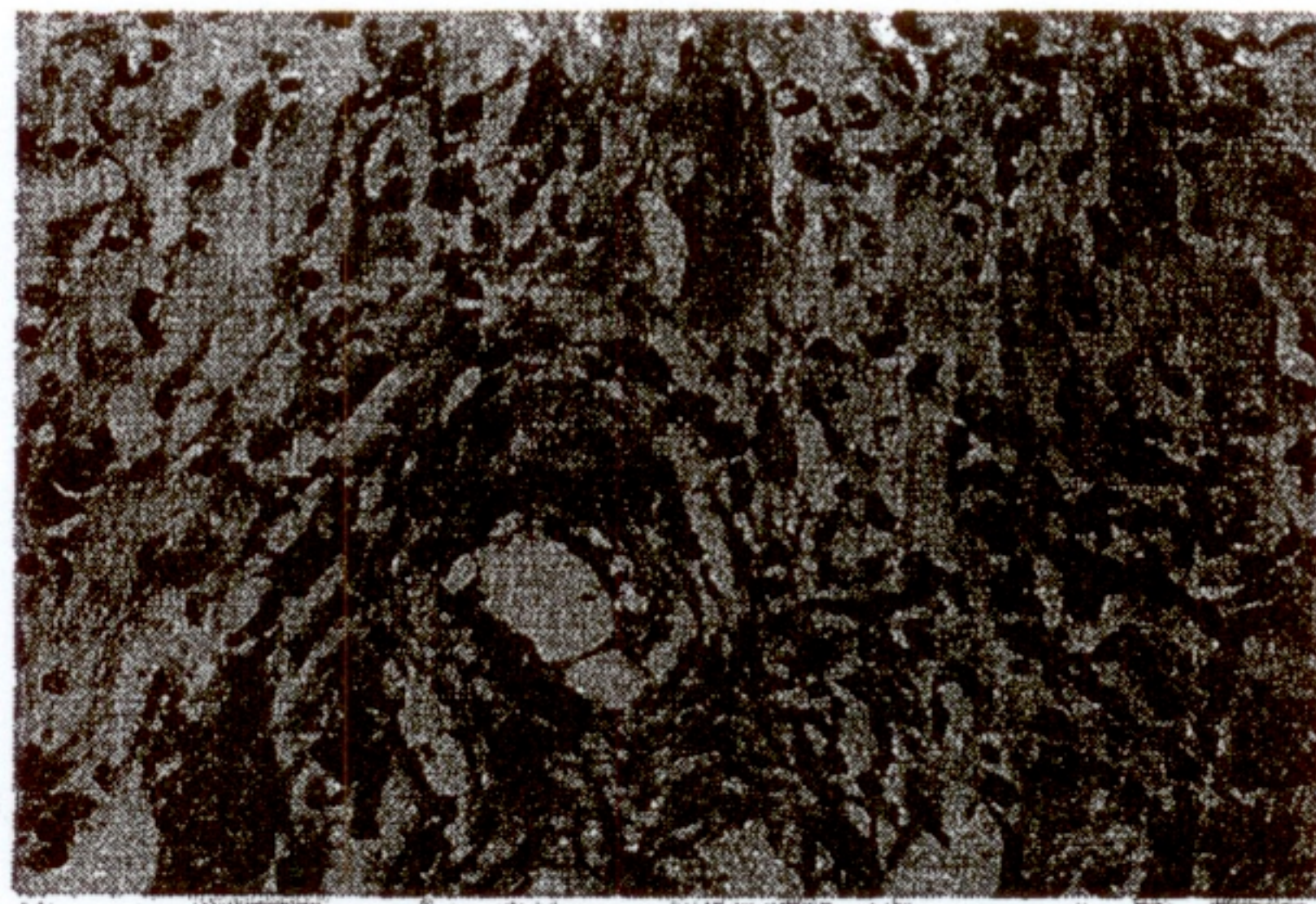

Fig. 7　Microfibrillar collagen 14 days after subcutaneous implantation shows strong invasion by inflammatory cells (180× magnification).

tested collagen materials. Seven days after implantation, the collagen fleece implant had an amorphous texture, gelled and induced minimal cellular reaction without encapsulation (**Fig. 4**). At the same time, the microfibrils were easy to identify, the implant was surrounded by a cellular capsule and inflammatory reaction with cells infiltrating the matrix of microfibrillar collagen (**Fig. 5**). At 14 days the cells were evenly invading collagen fleece (**Fig.6**) with the cellular reaction to collagen fleece being always less than that to microfibrillar collagen implants (**Fig. 7**). Both implants were infiltrated by inflammatory cells, granulocytes and macrophages. Few fibroblasts (fusiform shaped cells) adhered to the surface of microfibrillar collagen fibers, which were still easy to identify. At 28 days the identification of collagen fleece was difficult and the presence of the original implant was made by the residual presence of tissue atypical cells and some deposits of newly formed collagen. This is shown for collagen
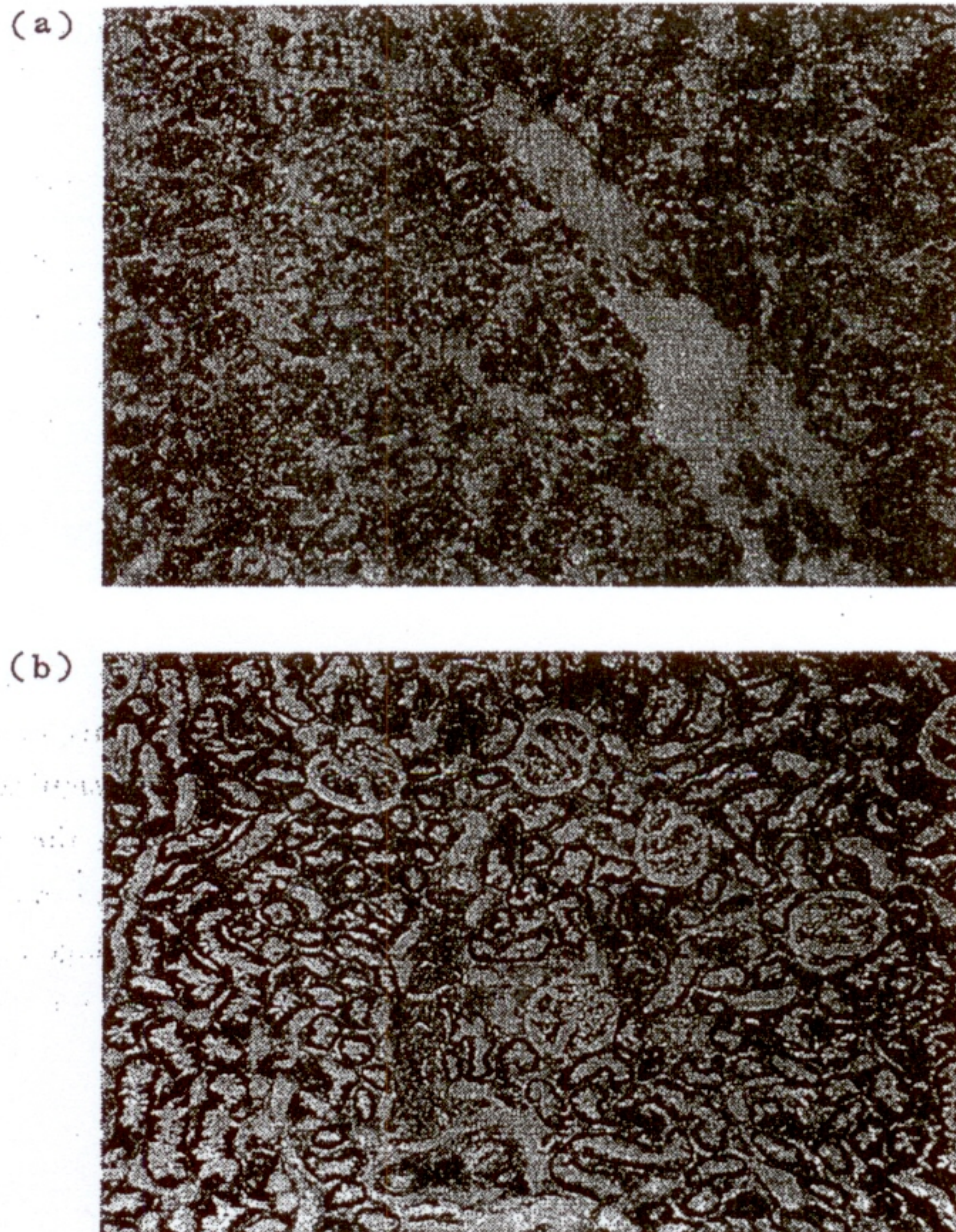
（a）

（b）

Fig. 8　At 28 days only few, small foci with collagen fleece implant can be detected in the spleen (a) or in the kidney(b) (120× magnification).

fleece subcutaneous and intrarenal implant (**Fig.** 8 a, b). At this time, microfibrillar collagen implant was minimally changed in size, was infiltrated by mixed cell population and invaded by microvessels. At two months collagen fleece placement was guessed by the remaining presence of abnormalities in tissue morphology, showing few inflammatory cells and some collagen deposit. At the same time, microfibrillar collagen was easy to identify although the volume of the implant was reduced by 60–70%. Only sporadically we found the presence of giant cells in either collagen implant. There was no abnormal accumulation of lymphocytes, mast cells or plasma cells.

3 Semiquantitative scorring of biodegradation of collagen fleece vs microfibrillar collagen

Two to five implants of either collagen fleece or microfibrillar collagen into five different tissues were scored by two investigators for the above shown parameters. Analysis of these data showed a certain similar pattern of the tissue response to either collagen fleece or microfibrillar collagen, regardless of the implanted tissue. It was also observed that the evaluation of

Table 3 Summary of the semiquantitative evaluation of tissue reactions to implanted collagen hemostats

Parameter tested	7 days		14 days	
	Collagen fleece	Microfibrillar collagen	Collagen fleece	Microfibrillar collagen
Cell infiltrate density	2.7±0.3	2.4±0.25	2.3±0.41**	3.0±0.01
Capillary+New collagen	1.0	1.0	1.46*	1.04±0.09
Resorption	2.82±0.47**	3.90±0.1	2.2±0.18***	3.8±0.21

Each score was evaluated by two investigators.
Variability is shown by standard deviation.
Asterisks refers to statistically significant difference at $p < 0.05$(*),　$p < 0.01$(**),　$p < 0.001$(***).

tissue edema was difficult to assess and therefore this information is not included in further statistical evaluation, which posted all data on the given tissue reactivity parameter for a certain implant regardless of the tissue. A Student–t test was used to test the significant differences as shown in **Table 3**. The data indicate the most striking differences in the rate of resorption. The differences in the morphological picture became more pronounced in later postimplantation periods, obviously reflecting faster disappearance of collagen fleece implant. This information, although subjective and semiquantitative, supports the above presented biochemical results.

DISCUSSION

The results of this study, based on biochemical or morphological assessment of tissues implanted with two forms of collagen fibers, used commercially as topical hemostatic materials, show that collagen fleece (Novacol) is resorbed more than twice as fast as microfibrillar collagen (Avitene). The actual resorption time for collagen fleece appears to be 4 to 6 weeks; for microfibrillar collagen it is close to 3 months.

Collagen proteins contain a unique and most abundant amino acid, hydroxyproline (Hyp). Measurement of urinary excretion of the Hyp (free and in peptide form) has been extensively used as an index of collagen degradation[6]. This study showed that only 11 to 13% of all the biodegraded collagenous Hyp is excreted through the urine. This value is similar to observations of others:

Prockop suggested that only approximately 5–10% of the Hyp released by the degradation of insoluble collagen was excreted in the urine, the remaining 90–95% being metabolized to 1-pyrroline-3-hydroxy-5-carboxylic acid, CO_2 and urea[7]. Woessner found that less than 15% of the Hyp of degraded collagen appears in the urine of the post partum rat[8]. In experiments by Weiss and Klein 20% of the administered radioactive hydroxyproline peptides was recovered as urinary Hyp[9].

Among factors known to affect the urinary excretion of Hyp under normal conditions are diet, age-growth, sex and body size[6]. None of these factors should be of any importance

28 days		60 days	
Collagen fleece	Microfibrillar collagen	Collagen fleece	Microfibrillar collagen
2.1±0.24**	2.9	1.1±0.11***	2.7±0.37
1.8±0.35**	1.1±0.15	1.2±0.2**	1.8±0.17
1.3±0.22***	3.1±0.42	1.0***	2.5±0.26

in our experimental design, as the diet, age, sex and size of the rats were identical. Thus, the reported results reflect the physico-chemical nature of the tested collagen material.

Biochemical data on urinary Hyp excretion clearly showed two differing patterns between collagen fleece and microfibrillar collagen implants: early postimplantation difference (1 to 4 days) shows almost five times more Hyp being excreted in collagen fleece implanted rats. We found that collagen fleece is more chemically degraded by extensive purification processes than microfibrillar collagen, which is reflected in lower shrinkage temperature (T_s) for collagen fleece. This also explains the gelling capacity of collagen fleece in contact with tissue fluids. In our previous publications[10, 11], we showed the importance of this gelling phenomenon in achieving hemostasis. Collagen fleece gels quickly in contact with biological fluids to form a homogenous-colloidal milieu with considerable viscosity and gluing properties. Microfibrillar collagen remains more particulate.

The late postimplantation difference points to a significantly faster trend to achieve the baseline of urinary Hyp levels in collagen fleece implanted rats than in microfibrillar collagen implanted rats.

Another finding to be discussed is the different morphology of the implantation sites for collagen fleece and microfibrillar collagen. These analyses were done at various postimplantation times up to 10 weeks after implantation of the collagen materials in the rats. The histological evaluation showed quite distinctly more cellular infiltration and more new collagen deposition with the implanted microfibrillar collagen; in many instances microfibrillar collagen implant was still detectable at this time. This is in contrast with the smaller cellular reaction to collagen fleece, which was not directly identified within the implanted tissues after 4 weeks and its presence was assumed from abnormal tissue morphology.

In considering the clinical significance of the rate of biodegradation of a collagen hemostatic material, keep in mind that neither too fast or too slow resorption is desirable. Fast biodegradation of collagen implant releases collagen molecules and peptides with known chemotactic properties[1]. An acute inflammatory reaction with edema and seroma results. Too slow resorption of collagenous implants provides a structure for late fibrotic encapsulation, adhesion formation. If the collagen implant is resorbed within one week (regardless of implantation

site), this is too fast. On the contrary, resorption extended over three months should create fibrotic reactions, specifically the formation of fibrotic adhesions. From this discussion it follows that collagen fleece is resorbed within one month, and these dynamics were considered optimal.

It is worth mentioning, however, that there are medical uses of highly cross-linked collagen, which resorbs very slowly within several months (6–9 months). In these situations, the collagen implant serves as a scaffold for ingrowing connective tissue, replacing damaged tendon, ligament, bone or cartilage[1].

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