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Mesothelial Regeneration in the Rat and Effect of Urokinase

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Introduction

The mechanism of development of adhesion in the intestines is generally regarded as attributable to appearance of fibrinogen due to the inflammation caused by the damages inflicted on the serosal surface by bacterial, physical, or mechanical factors or foreign matters, and consequently to the conversion of fibrinogen into fibrin owing to the same mechanism as in the blood clotting and subsequent appearance of fibroblasts, which accelerate organization of the cells into connective tissues. However, a number of points remained unclarified so far with the factors exerting influences upon the above processes and also the details of the ensuing results[1)-3)].

It is considered that, unlike the recovery from damages of skin or mucosa, that of the damages of serosa is initiated from the edges of the lesion, and that the mesoblastic tissue is the source of the regenerated mesothelial cells. However, some reports have been recently published, which describe that a variety of macrophages are observed on the surface of lesions both with a light microscope and an electron microscope since the early stage of their development, and that the macrophages and the source of the newly regenerated mesothelium, but it seems that there have been no firm evidence for these reports[4)-10)].

Furthermore, a number of experimental or clinical studies have so far been carried out concerning the methods for prevention of adhesions in the intestines or the agents which are effective for prevention, but the fact is that there have been no method, which can satisfactorily prevent adhesion. We, therefore, carried out histological observations on the progress in the recovery from abrasion of the serosal surface of the intestines and the influences of urokinase on the progress, which is claimed to be effective for preventing adhesion. Furthermore, we measured the fibrinolytic activity of the serosa and examined the relationship between the adhesion and the fibrinolytic activity. We further investigated whether or not administration of urokinase is effective for preventing adhesion.

Method

Male Wistar rats each weighing 250-300g were employed for this experiment. The rats were anesthetized with ether and shaved with a razor on their abdomen. The subsequent

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processes were proceeded as cleanly as possible. In order to minimize troubles by the surgical operations, and to prevent haemorrhage as far as possible, the laparatomy was carried out with a minimum median incision on the abdomen. The surface of the serosa was strongly rubbed with a piece of dry gauze over the nearly whole serosal surface of the caecum and spots of haemorrhage were produced on it. The animals were divided into a group with abrasion alone and a group with the abrasion immediately followed by intraperitoneal administration of 600 IU/0.1kg of urokinase (a product of Green Cross Co., Ltd., tradenamed as Urokinase®)

Twenty rats with abrasion alone and another twenty rats with administration of 600 IU /0.1kg of urokinase after abrasion were prepared, and these were subjected to laparotomy again under anesthesia with ether after two weeks. They were classified in accordance with the classification method by KNIGHTLY[11] in terms of the degrees of grossly inspected adhesion, and the two groups were comparatively evaluated.

Each of the two groups was sacrificed after intraperitoneal perfusion with 2% glutaraldehyde-phosphate buffer solution under anesthesia with ether before abrasion, immediately after abrasion, and 30 minutes, 1, 3, 6, 12, and 24 hours, and 3, 5, 7, and 10 days after abrasion. Each two rats were used in each time. Then, specimens from the intestine were collected, which were stained with H. E. and Toludin Blue for observation by a light microscope and scanning and transmission electron microscopes.

The observation with the scanning electron microscope was carried out by fixing the specimen with 2% glutaraldehyde-phosphate buffer solution (pH 7.4), dehhydrating the fixed specimens with a series of dilutions of alcohol, replacing them with isoamyl acetate, drying with the critical drying apparatus, double coating with carbon and gold, and observating them with the scanning electrom microscope, Model SSM-2 of Hitachi, with an acceleration of 10 KV.

The observation with the transmission electron microscope was performed by fixing the specimen with 2% glutaraldehyde in phosphate buffer solution with a pH of 7.4, refixing them with 1% OsO_4 and embedding them into Epon after dehydrating with alcohol. The ultrathinnly sectioned specimens were stained with uranyl acetate and lead citrate and observed with the electron microscope, Model Hu-11Ds of Hitachi.

Measurement of fibrinolytic activity : The fibrinolytic activity of the serosa was measured in accordance with the fibrin plate method, by using the unheated (standard) plate and heated plate. The specimens were collected as a piece of tissue of a round shape of 3 mm in diameter before and immediately after abrasion of serosa of caecum. The specimens were put on fibrin plate and the activity on the basis of dissolved area after 18 hours' incubation at 37°C on the plates was measured.

Experiment on the origin of mesothelial cells : In order to clarify the cells which are the origin of the regenerated mesothelium, 600 IU/0.1kg of urokinase and 0.5ml of indian ink were intraperitoneally administered for successive three days, and observation with a light microscope (with H. E. staining) was carried out with the lapse of time.

Results

Macroscopic observation : Table 1 indicates the degrees of adhesion in the range of 0°-4° on the basis of Knightly's classification. It was observed that 95% of the group with abrasion alone indicated strong adhesion of 3°-4°, while 78% of the group with urokinase showed adhesion less than 2°, which lighter than the above group. Two of the 20 rats of the urokinase group died, whose causes of death were identified as pneumonia upon autopsy.

Table 1

grade / No.		0°	1°	2°	3°	4°
Control group	20	0	0	1	11	3
Urokinase treated group	18	1	3	10	3	1

Classification of KNIGHTLY et al. (1962)

Grade 0 ; Complete absence of adhesion.
Grade 1 ; A single thin easily separated adhesion.
Grade 2 ; Less extensive but weak adhesion which withstand traction poorly.
Grade 3 ; Numerous extensive visceral adhesions without visceroparietal extension.
Grade 4 ; Numerous extensive dense adhesions which involved the adjacent mesentery intestine, caecum and extended to the abdominal wall.

Microscopic observation : The comparative observation with the lapse of time with an electron microscope on the two groups with abrasion alone and with urokinase after abrasion carried out. The surface of normal serosa is shown in Figure 1 and has long and fine microvilli densely covering that. Because of the dense covering, the cells of mesothelium can not morphologically be clarified, except a few fusiform swellings which were deemed to be the nuclei.

Immediately after abrasion of the serosa with a piece of dry gauze, no microvilli were noted on the surface, and the cells of the mesothelium were not shown. No basal membrane was either noted on the surface, and the lower layers were of a fibrous structure which was deemed to be connective tissues, indicating as a whole a constractive pattern composed of numerous fine grooves and some swellings. (Fig. 2)

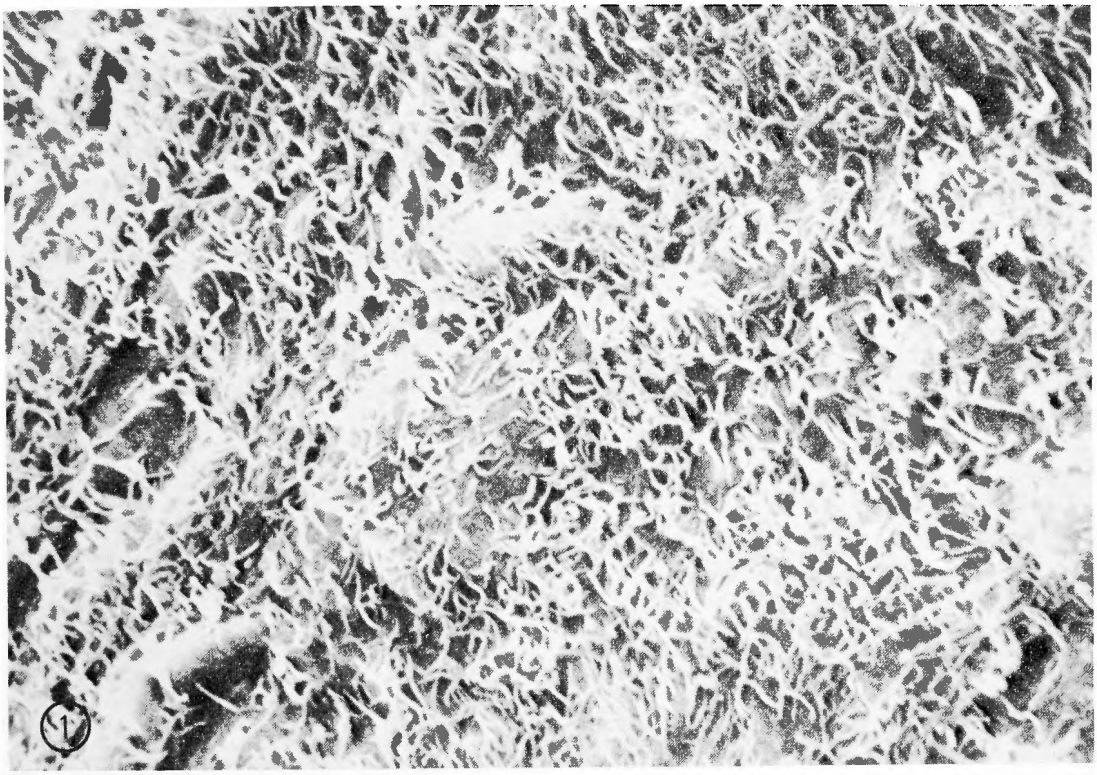
In the group with abrasion alone, release of erythrocytes and fibrin was clearly noted 30 minutes after abrasion, and fibers of the fibrin were of a relatively rough reticular structure (Fig. 3). Until 3 hours after abrasion, the fibers were adhered to each other gradually growing into thick fibers as time elapsed, and the reticular structure became finer and finer. At this stage, the surface of the serosa was found to be grossly turbid probably because of release of fibrin and seemed to be covered with the pseudomembrane, indicating the presence of slight adhesion of the adjacent intestines to each other. At 6 hours after abrasion, the membranous structure with fibrin came to cover all the serosal surface which were exposed, containing protrusions supposed to be erythrocytes. The fibers of the

connective tissues could not be found at all, and the surface was quite flat and smooth (Fig. 4). After 24 hours, protrusions of erythrocytes became gradually invisible because of release of fibrin, which formed a plate structure, so-called fibrinous aggregation (Figs. 5 a and b). Around the 5th day, the surface which had been flat and smooth gradually became rugged, and flecky round protrusions about 4.5 μ in diameter were uniformly detected (Fig. 6). On the 7 th day, the membranes formed by fibrin gradually disappeared, and the flecky protrusions underneath were exposed. The surface thus exposed was found finely divided with grooves which were deemed to be the crevices between cells. On the surface of the cells, short and fine villous structures were noted (Fig. 7). On the 10th day, the whole cells in irregular forms came to be exposed, indicating smooth fusiform shape and the crevices filled with needle-type protrusions. The surface of the cells was fully covered with well-grown fine villi, indicating a structure similar to that before abrasion (Fig. 8).

On the other hand, in the group with urokinase, little fibrin was found to have been released and the fibrin membranes as seen in the group with abrasion alone were not observed, indicating the exposed surface of the connective tissues from underneath covered with spherical and short microvilli ; these so-called macrophages were shown at some locations (Figs. 9 a, and b). At 12 hours after abrasion, macrophages gradually increased, covering around half of the exposed surface. However, with ample magnification, macrophages in spherical, fusiform, and flat forms were mixed each other (Fig. 10). At 24 hours, the surface became to be fully covered with these cells and the exposure of the connective tissues underneath was completely disappeared (Fig. 11). The macrophages were reduced gradually. On the third day, they were almost eliminated and the surface were covered with flattened cells and their surfaces were covered with short microvilli (Fig. 12). In some crevices of the cells, the connective tissues were shown to be exposed from underneath. On the 7 th day, the microbilli were found to be well developed and longer, covering the surface so densely that the outlines of the cells were not cearly seen. On the 10th day, the appearance was identical with that of the normal one.

Observation of fibrinolytic activity : The fibrinolytic activity of the normal serosa of the intestines before abrasion on the bases of lysis zone was $321.8 \pm 9.9 mm^2$ with unheated plate and $272.0 \pm 5.9 mm^2$ with heated plate. Immediately after abrasion of the serous membrane, the activities were respectively $215.4 \pm 18.7 mm^2$ and $182.1 \pm 14.1 mm^2$, indicating drop of the values (SE, n=31) (Figs. 13 and 14).

Origin of mesothelial cells : In the experiment in order to clarify the origin of the mesothelial cells, indian ink and 600 I.U./0.1kg of urokinase were administered after the abrasion of the serosa of the intestines, and laparotomy was again performed 24 hours thereafter. Indian ink was found to have been concentrated only at the location of the abrasion of the serosa, whereas little ink was found at other locations (Fig. 15). After the abrasion, the indian ink and urokinase were continuously administered for successive 3 days. The findings on the 8 th day after abrasion, when the regeneration of the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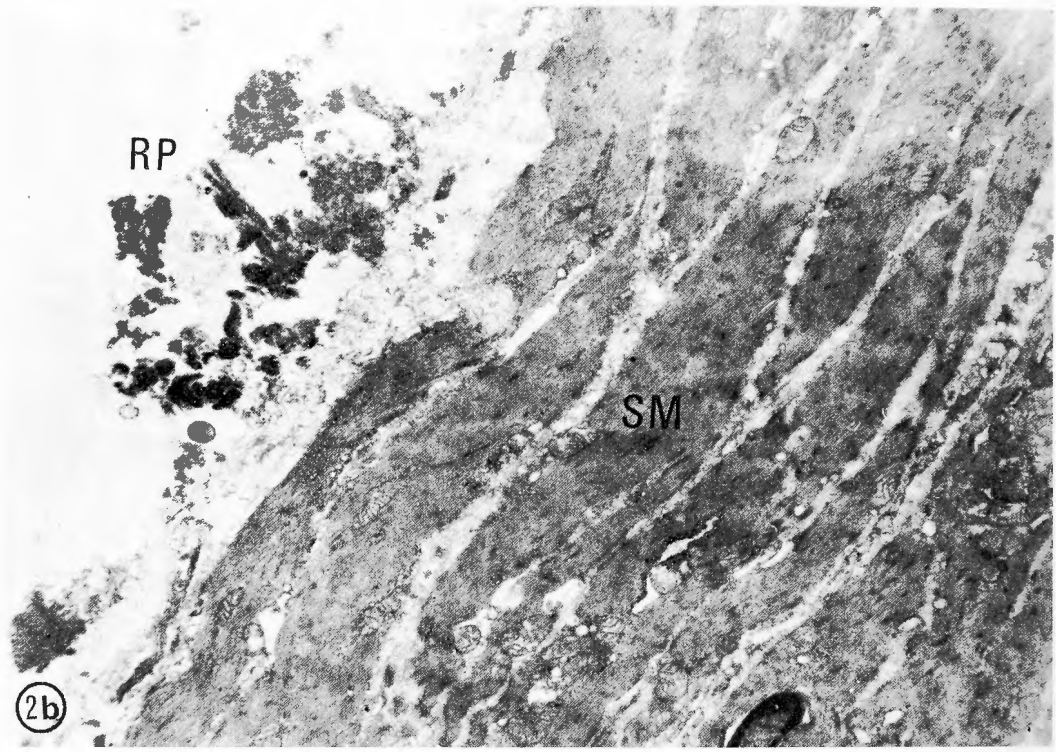
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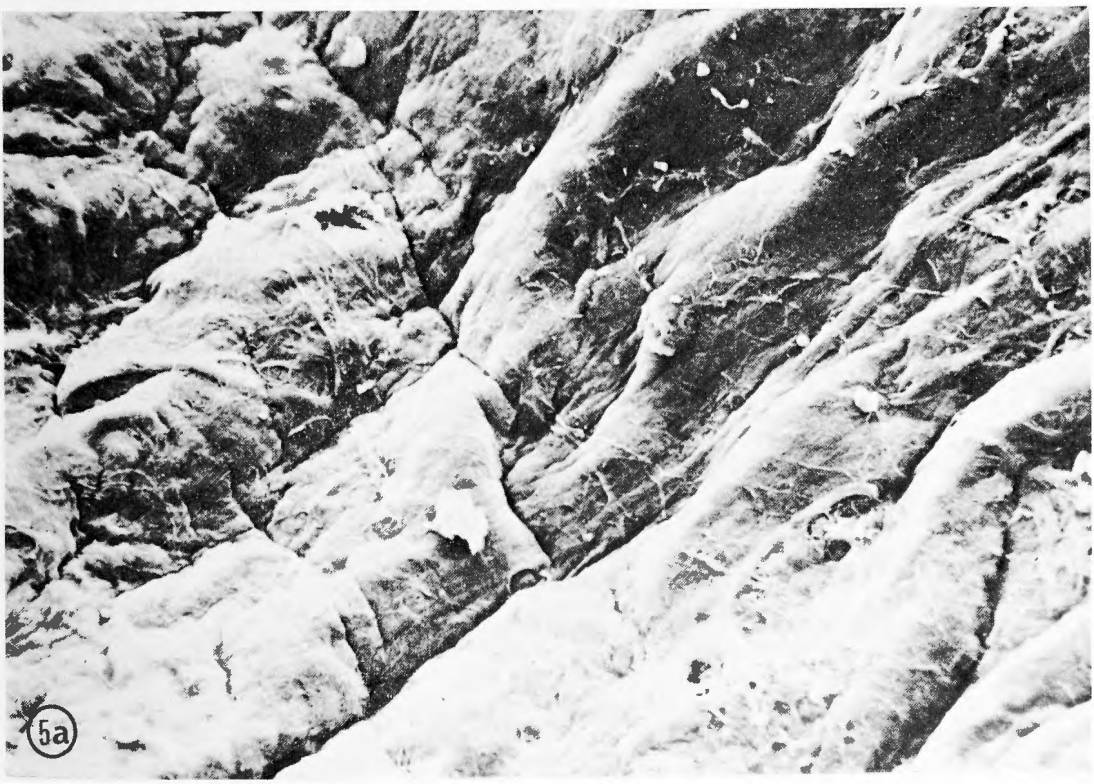
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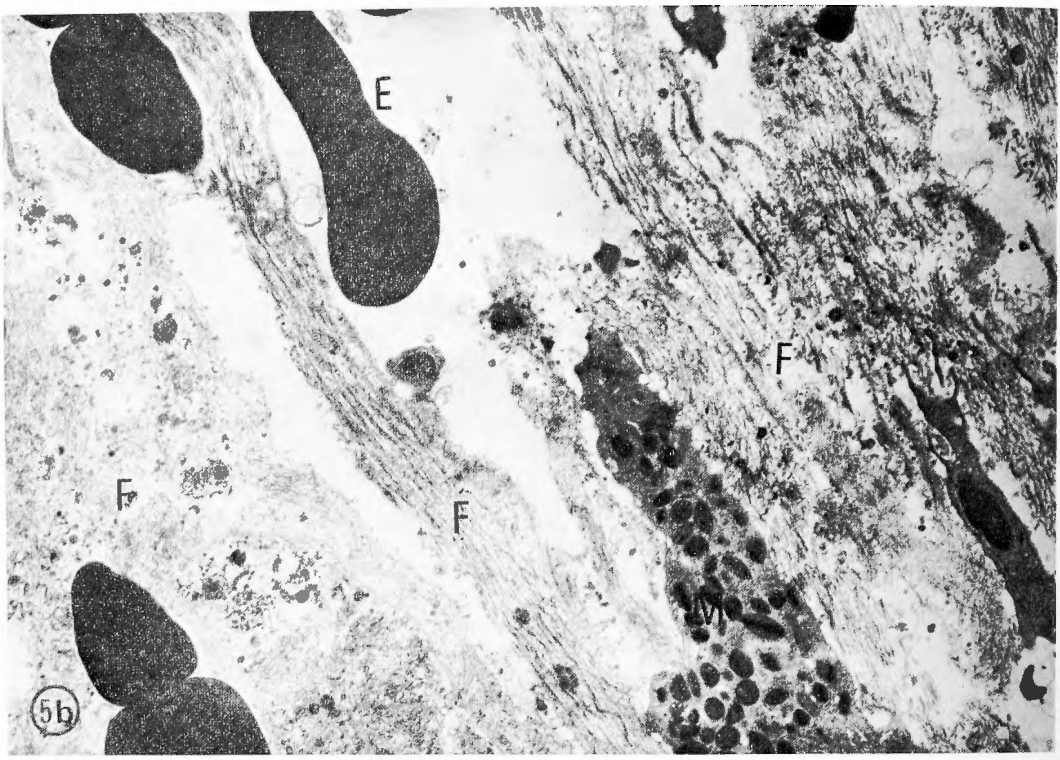
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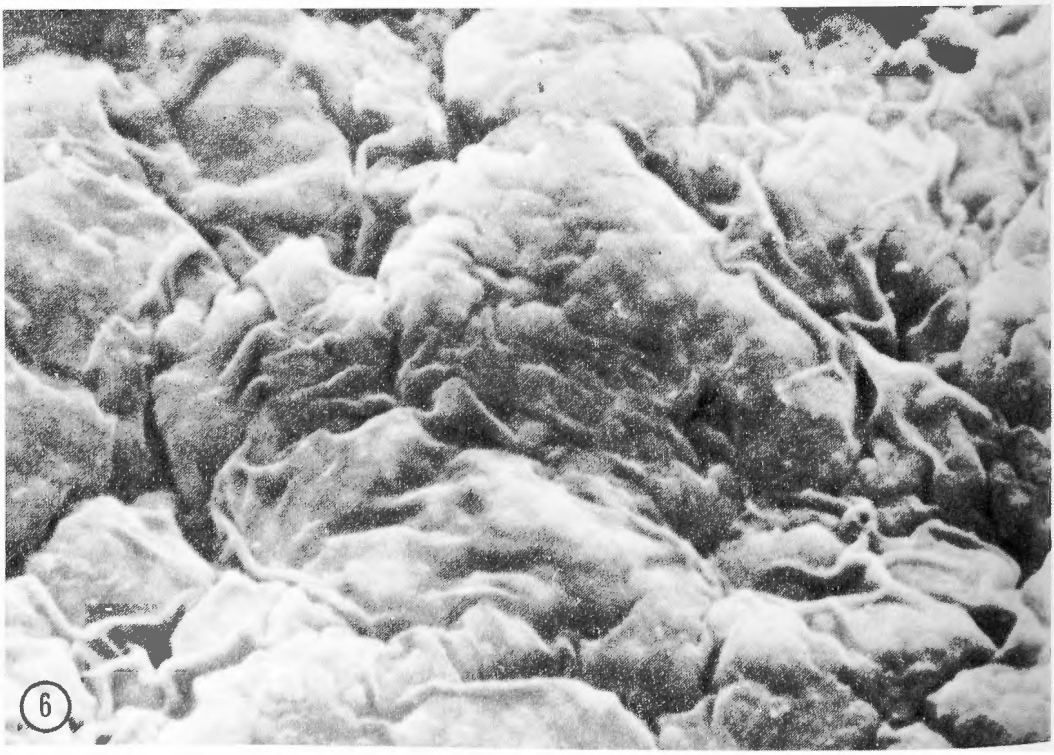
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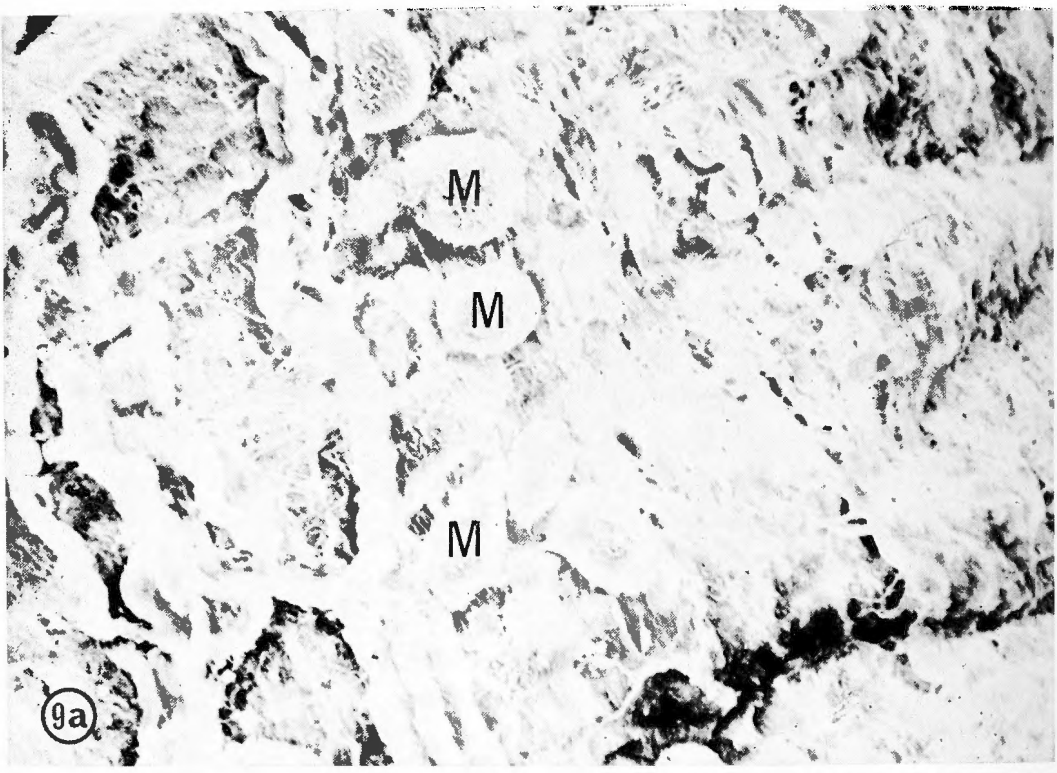
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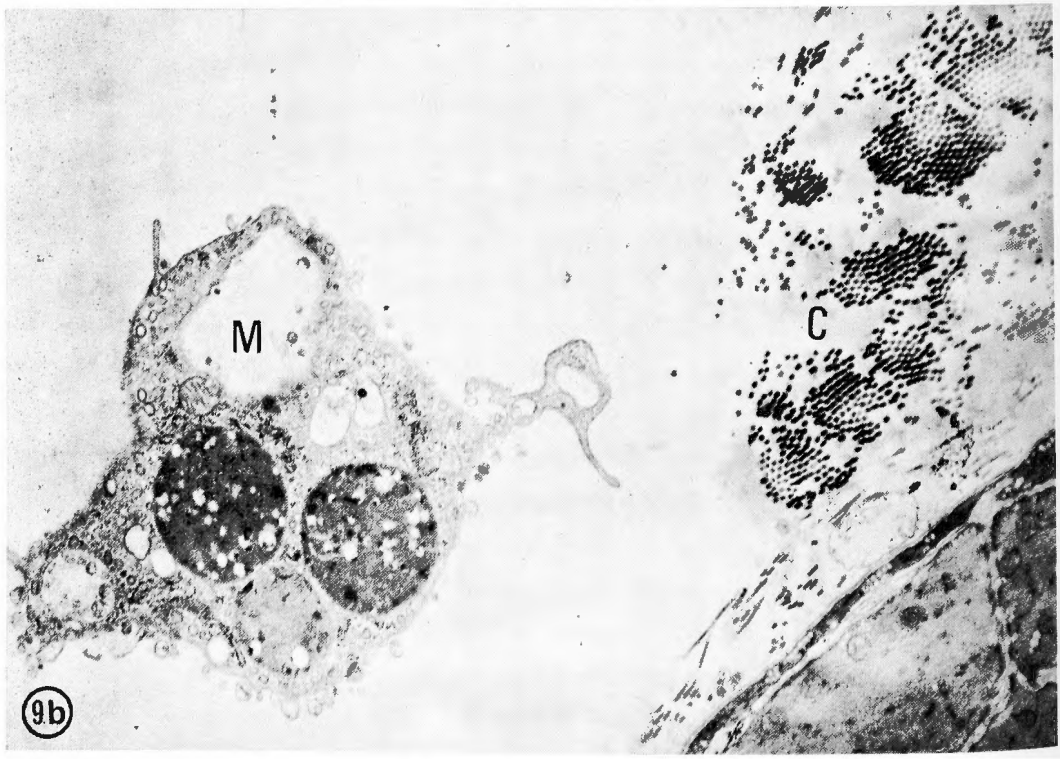
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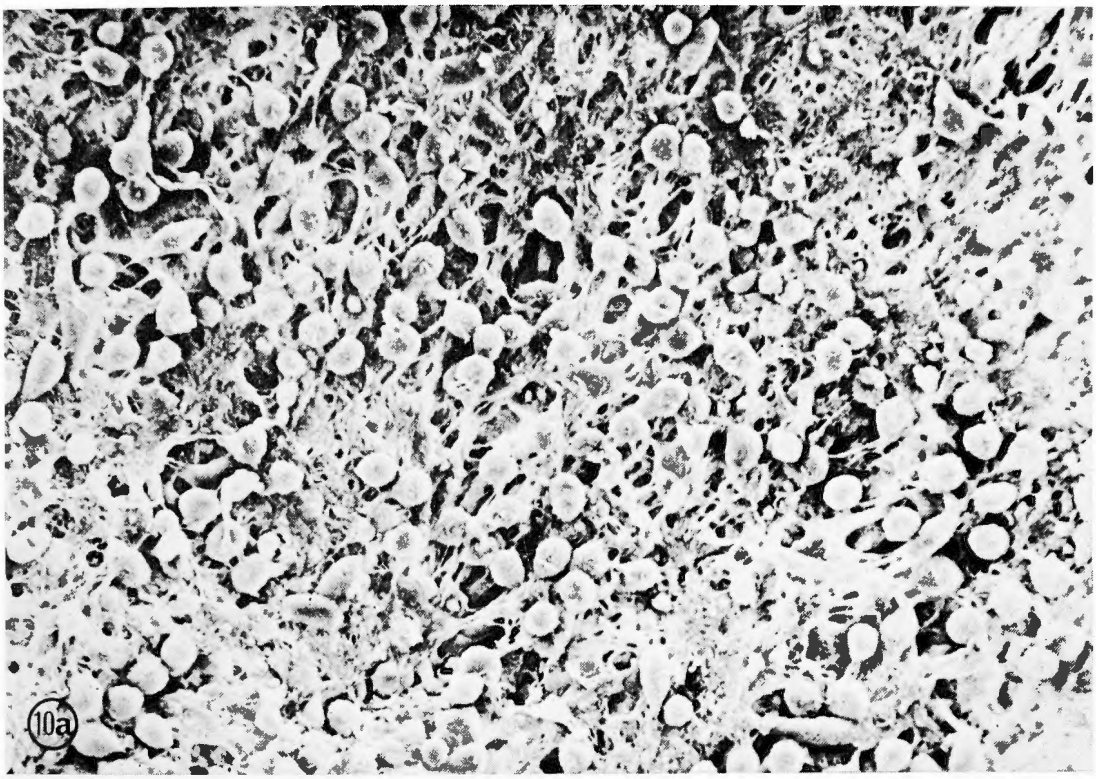
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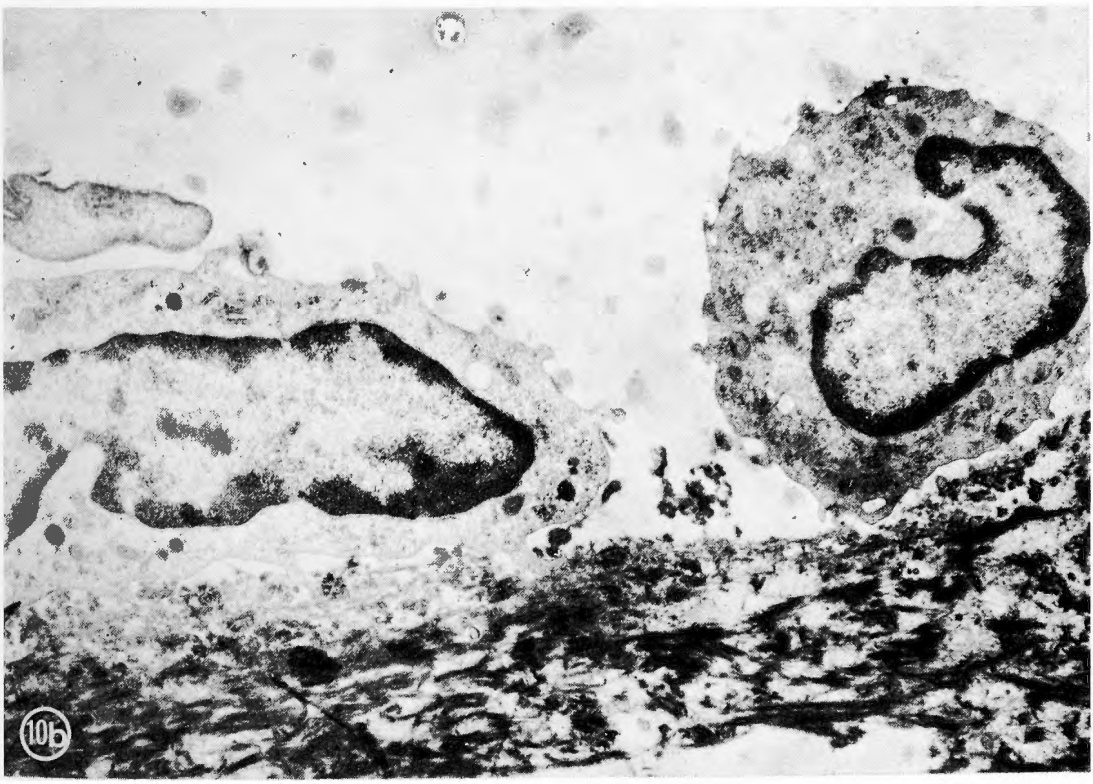
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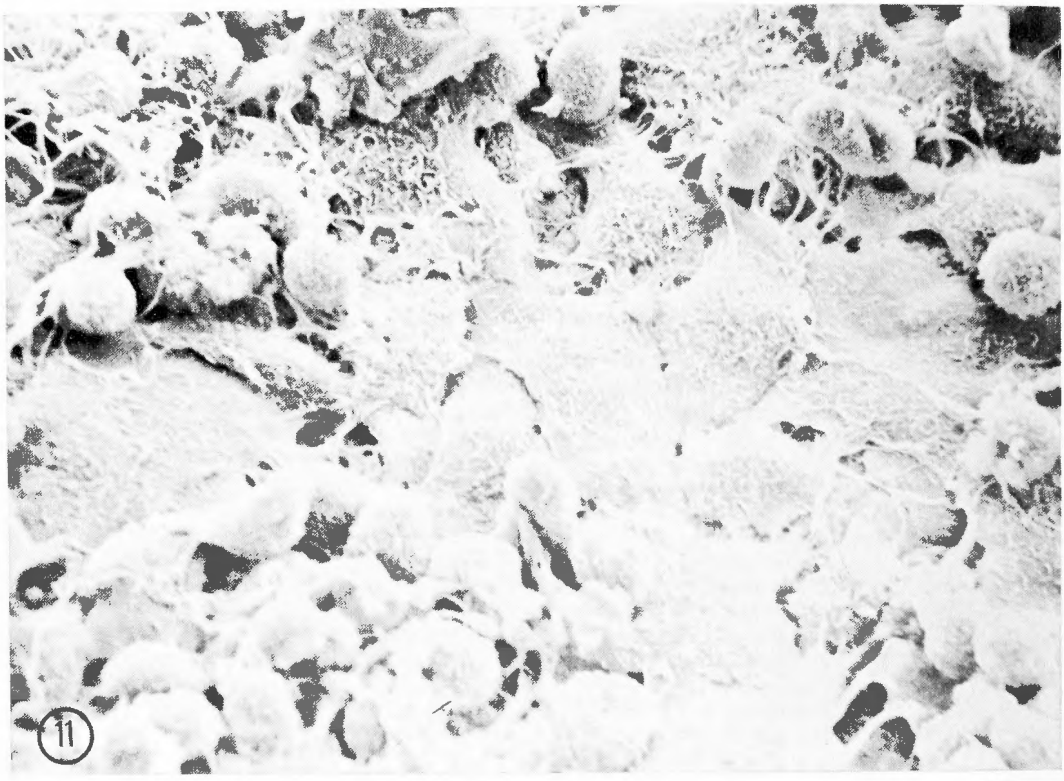
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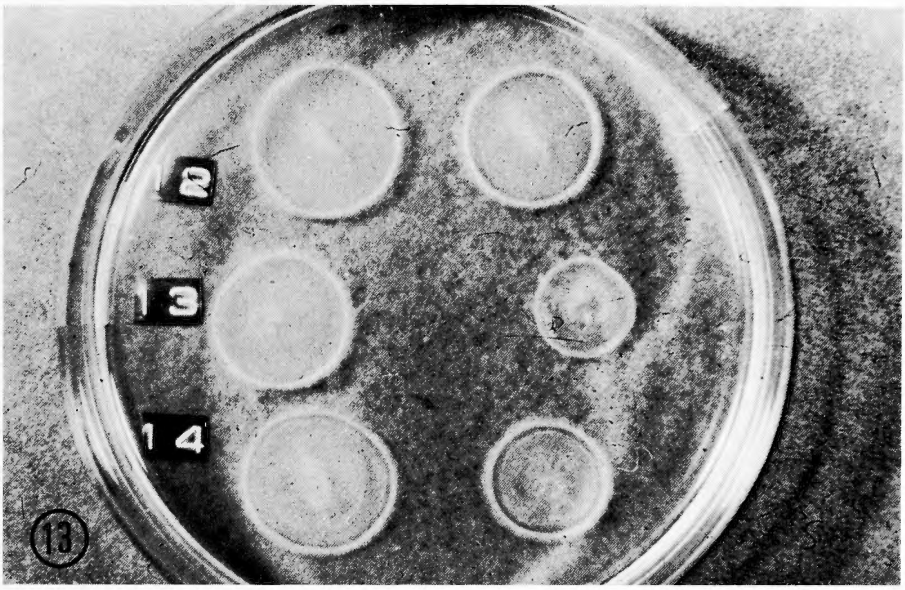
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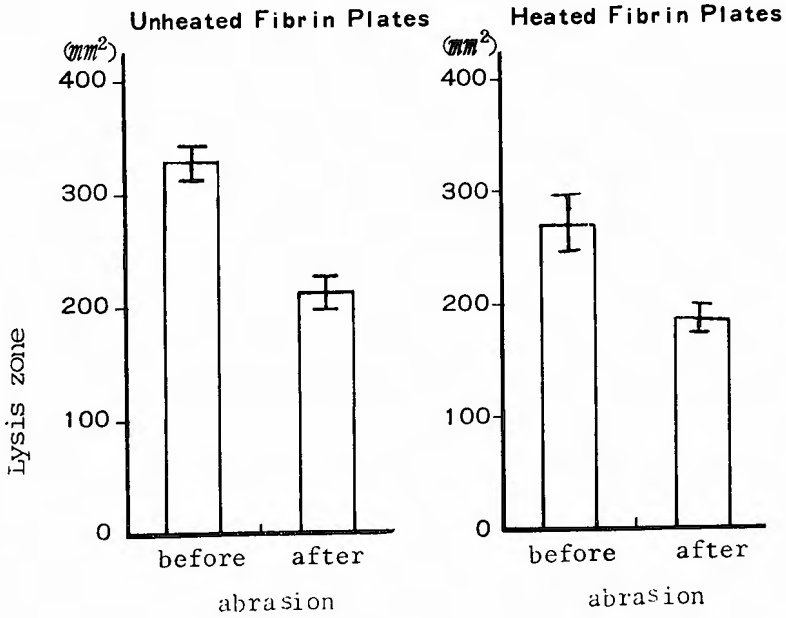
Unheated Fibrin Plates
Heated Fibrin Plates
(mm²)
400
300
200
100
0
Lysis zone
before
after
abrasion

Fig. 14

15

16

Fig. 1 Normal serosal surface of the rat caecum having numerous long microvilli. (×5,000)
Fig. 2a Naked surface immediately after abrasion of the serosa. The basement membrane is removed and the connective tissue are exposed. (×2,000)
Fig. 2b A transmission electron microscopic (TEM) photograph of the serosa immediately after abrasion. Mesothelial cells and the basement mambrane is removed. (RP); ruptured cell. (SM); smooth muscle. (×4,700)
Fig. 3 30 min. after abrasion of the serosa. Fibrin nets and erythrocytes are shown. (×2,000)
Fig. 4 6 hours after abrasion. The surface is covered with fibrin membrane and contains erythrocytes. (×2,000)
Fig. 5a 24 hours after abrasion. Fibrinous aggregation is shown. (×500)
Fig. 5b A transmission electron microscopic photograph of the surface 24 hours after abrasion. (E); erythrocyte, (M); macrophage, (F); fibrin bundles. (×4,700)
Fig. 6 5 days after abrasion. Numerous fleck structures are shown through the membrane of fibrinous aggregation. (×1,000)
Fig. 7 7 days after abrasion. Newly regenerated cells are exposed after disappearance of fibrinous membrane. (×5,000)
Fig. 8 Regenerated surface 10 days after abrasion. The surface is covered with newly regenerated mesothelial cells. (×1,000)
Fig. 9a 3 hours after abrasion and intraperitoneal injection of urokinase (600 I.U./0.1kg). The connective tissue are exposed on the surface, but several round cells which are considered to be macrophages are shown. (M); macrophages. (×2,000)
Fig. 9b A photograph of TEM of 3 hours after abrasion and treatment of urokinase. A single cell is shown near the surface. (C); collagen fibers. (M); macrophage. (×10,000)
Fig. 10a 12 hours after abrasion and treatment of urokinase. Spherical cells with short microvilli are increased in number. Spindle cells with same short microvilli are shown among those cells. (×1,000)
Fig. 10b A photograph of TEM of 12 hours after abrasion and treatment of urokinase. Macrophages adhere on the surface. (×4,700)
Fig. 11 24 hours after abrasion and treatment of urokinase. Numerous spherical cells, spindle cells, and flattened cells are mixed. (×2,000)
Fig. 12 3 days after abrasion and treatment. Spherical cells are not shown. The surface is covered with flattened cells having numerous microvilli. (×1,000)
Fig. 13 Fibrinolytic activity is indicated by the area dissolved the fibrin plates. The left is before abrasion. The right is after abrasion.
Fig. 14 Comparison of fibrinolytic activity before abrasion with that after abrasion.
Fig. 15 24 hours. after abrasion of the serosa and intraperitoneal injection of indian ink and urokinase. Concentration of indian ink is shown on the abraded portion alone.
Fig. 16 8 days after abrasion intraperitoneal administration of indian ink and urokinase. Uptake of indian ink is shown in the newly regenerated mesothelial cells (arrows). (×200)

mesothelium was supposed to have been completed, were as shown in Figure 15, in which uptake of the ink into the cells of the mesothelium thus regenerated was noted (Fig.16).

Discussion

A number of studies have been carried out since many years ago on the development of adhesion on the intestines and the means for preventing it. A variety of experimental methods were attempted by many investigators for producing the adhesion of the intestinal tracts, such as abrasion of the serosa[1], drying and consequent hemorrhagia[3], oligemic state of the intestine[12),13], and introduction of foreign substances[1].

SAKAKIBARA[1] reported that, after abrasion of the serosa, the stimuli reached some of the muscular layers, which almost surely caused fibrous adhesion. Therefore, we carried out this experiment using the method proposed by SAKAKIBARA. However, the degrees and speed

of the regeneration varied with the strength of abrasion ; the lighter the lesion, the faster the time for the regeneration. The regeneration was faster in the intestines which still retained the basal membrane.

Anatomically, the peritoneum is composed of the serosa and the parietal peritoneum, and the surface of the serosa is covered with a monolayer of mesothelial cells. A number of studies have so far been reported on the regeneration of the parietal peritoneum ; in 1966, ESKELAND[7] reported that the surface of lesion, however wide it may be, was covered completely with newly regenerated mesothelium by the 8th day, in his experiments with strong lesion including the connective tissues underneath, which was found out with a light microscope and an electron microscope. HUBBARD etal.[14]. histologically investigated the processes of recovery of the lesion on the parietal peritoneum of rats, rabbits, and dogs, and reported that the regeneration of the mesothelial cells starts with the metamorphosis of the in-situ mesothelial cells and not with the cut edge, and that the whole area of the lesion was recovered uniformly, irrespective of the size of the lesion. The origins of these mesothelial cells have been considered to be the surrounding peritoneum, the connective tissues from underneath, and the cells in the peritoneum, but none of them have been fully confirmed to date. JOHNSON et al.[9] reported that the recovery can be advanced by the implantation of the intraperitoneal mesothelial cells. ELLIS, HARRISON, and HUGE[5] reported that the lesions on the parietal peritoneum are mainly replaced with fibroblasts into mesothelial cells. MATSUSE et al.[15] reported that the tissue macrophages are transformed into the mesothelial cells. However, ESKELAND[6),7] suggested that the free floating macrophages are the important source of mesothelial regeneration of the lesions on the parietal peritoneum and reported that the macrophages were found with an electron microscope to be very slowly converted into the mesothelial cells. On the basis of our experiments, it can be claimed that our findings coincided with the reports thus published so far which had reported that fibrin is released together with the exudates in the same mechanism as with the blood clotting at the early stage after the lesion of the serosa of the intestines, and that the fibrinous aggregation can take place very gradually. It was found that the formation of fibrin as seen at the early stage after the lesion could be prevented by the administration of urokinase, that no fibrinous aggregation was consequently found due to the ensuing dissolution of fibrin, that the release of macrophages takes place at the early stage on the surface of the lesion, which indicate formation of mesothelial cells as early as 12 hours after the lesion, and that there elittle release of macrophages and the mesothelium was regenerated from the layer underneath the fibrinous aggregation. The former was almost regenerated within about 3 days, whereas it took about 10 days for the latter to be regenerated. It was therefore inferred that the difference was derived from the possibility that blood clotting and fibrin could retard the regeneration. It was also inferred that the origin of the mesothelialization on the lesion of the serosa was the free floating macrophages. Following intraperitoneal injection of the indian ink and urokinase after abrasion of the serosa, the concentration of the indian ink was found only at the location of abrasion when relaparotomy was carried

out 24 hours after infusion. This would be attributable to the possibility that the macrophages in the intraperitoneum phagocytized the particles of the indian ink and then were adhered to the surface of the lesion. On the 8th day when the mesotheliun was supposed to have been regenerated, laparatomy was performed and the observation with a light microscope indicated that the uptake of the ink was noted in the cells of the mesothelium thus regenerated. On the basis of these findings, it was assumed that the free floating macrophages phagocytize the particles of the indian ink, which adhere to the surface of the abrasion, playing a role also in the regeneration of the mesothelium.

As detected in the findings with an electron microscope immediately after abrasion of the serosa, the serosa had no mesothelial cells and the connective tissues from underneath were exposed, and the fibrinolytic activities of both the non-heated and heated plates were found to have been lowered. This finding suggested that the presence of mesothelial cells maintains the fibrolytic activity contributing to prevention of adhesion, because of the reports by HARTWELL and GERVIA[16] that the adhesion can be prevented by the fibrolytic ability and the epithelial functions possessed by the cells of the serosa. The adhesion as strong as 95% as seen in our experiments would be attributed to the drop or elimination of the above-mentioned preventive mechanism of the mesothelial cells. It can be also inferred that after administration of urokinase, the release of fibrin was prevented and the fibrin was dissolved accelerating regeneration of the mesothelium at the early stage and maintaining the fibrolytic ability and epithelial functions. This hypothesis can be also suggested from the results of our experiments that 78% had little or no adhesion.

Summary

The following experiments were carried out in order to clarify the pathophysiology of adhesion of the intestines. Following intentional abrasion on the serosal surface of caecum, the rats were divided into the group with single intraperitoneal administration of 600 I.U./0.1kg of urokinase immediately after the abrasion and the group without its administration. The processes of the recovery from the damages in both groups were observed with the lapse of time with a light microscope, transmission, and scanning electron-microscope.

In the non-medicated group with abrasion, the recovery was retarded due to the presence of fibrin and blood clots, and it took about 10 days for the recovery, whereas, in the group with urokinase, occurrences of fibrin were less, and consequently, mesothelium could be regenerated relatively quickly as macrophages became to be released. The recovery was not initiated from the cut edge as seen in the epithelialization of the skin, but the recovery of the whole lesion from the damage was uniformly and quickly proceeded at the same time. It has been reported so far that the main sources of the newly developing mesothelium is the in situ mesothelial cells, fibroblasts, or various macrophages, but we suggested that the new mesothelization is derived from the freefloating macrophages.

The fibrinolytic activity of the serosal surface was measured with the fibrin plate method to find that the activity was reduced in the damaged serosa causing frequent

adhesions, whereas administration of urokinase was found to lower the frequency of the adhesions even on the serosa whose activity had been reduced by the damage.

On the basis of the above findings, it was assumed that the fibrinolytic activity possessed by the mesothelial cells is of a prophylactic mechanism against adhesion and that lack of the activity could induce the adhesion, whereas urokinase could maintain the fibrinolytic activity even under such a condition, playing an important role in prevention of the adhesion.

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和文抄録

ラットにおける漿膜再生機構と Urokinase の効果

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腸管癒着の病態生理を解明する目的で，まず漿膜の再生機構について観察した．ラット盲腸部の漿膜面を擦過し損傷を与えた後，擦過単独群と擦過直後 fibrinolysin 600 I.U./0.1kg を腹腔内1回投与群に分け，経時的に光顕，透過および走査電顕にてその修復過程を観察した．

擦過単独群では修復は fibrin や血液凝固塊により遅延され，約10日間を要するのに対し，fibrinolysin 投与群では fibrin の析出が少なく，macrophage の遊出とともにかなり早期に中皮の再生がみられた．これらの修復は皮膚の上皮化のように創縁からではなく，欠損創全体が一様に速やかに行われた．なお，新生中皮の主源泉は in situ mesothelial cell, fibroblast あるいは種々の macrophage との報告もあるが，われわれの観察結果からは free floating macrophage 由来と考えられた．

また漿膜細胞のもつ線溶活性を fibrin 平板法により測定した結果，損傷をうけた漿膜ではその活性が低下し，癒着を若起する率が高いが，線溶活性を高めるといわれている urokinase の投与により，損傷をうけ活性が低下した漿膜においてさえ癒着の生じる率は低かった．このことより漿膜細胞のもつ線溶能は癒着防止の予防機構であり，その欠除により癒着が若起せしめられるが，urokinase はこのような状態でさえ，線溶能を維持させ癒着防止に役割を演じていると考えられた．