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Role of Polysaccharides Extracted from Human-type *Mycobacterium tuberculosis* in the Collagen Proliferation and the Suppression of Cancer Growth : From a Point of Pathobiological View

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ABSTRACT. The antitumor activity of Specific Substance Maruyama (SSM) consists of the activation and intensification of collagen proliferation in response to carcinoma. Experiments *in vitro* and *in vivo* (xenografts) indicated that SSM did not directly damage cancer cells, but that it did accelerate the proliferation of collagen fibers of the stroma. These collagen fibers enclosed the cancer cells, preventing their proliferation. Also, SSM remarkably accelerated the proliferation of collagen fibers under a specific or nonspecific immune condition. A basic activity of SSM thus appears to be the stimulation of the proliferation of collagen fibers in stroma invaded by cancer cells. The small blood vessels, blood capillaries, muscle fibers, and nerve fibers are partially composed of collagen. Furthermore, the biosynthesis of collagen seems to be accelerated by SSM in the carcinomatous necrotic lesions — a process that may also occur in fibroblasts, endothelial cells, and the extracellular matrices and cancer cells.

Key words : human-type *Mycobacterium tuberculosis* — antitumor activity — collagenation — cancer envelopment

SSM (Specific Substance Maruyama), a polysaccharide component (arabinomannan being the main component extracted from human-type *Mycobacterium tuberculosis*, Aoyama strain) is at present being used as a clinical test agent against cancer. Since its development as an anti-tuberculosis drug in 1944, SSM has had a distinct effect on stubborn skin tuberculosis.[1] While using this drug Maruyama, its developer, noticed a low incidence of cancer among patients suffering from tuberculosis and leprosy and was the first to report on its anticancer effect. Many investigators were unable, however, to present convincing experimental evidence concerning the antitumor mechanism of SSM in spite of numerous clinical test cases. But it did attract attention when significant biological effects, i.e., promotion of interferon production by activating lymphocytes and macrophages,[2] recovery of lowered lymphocyte activity in cancer patients (Yanagi, T.) and disappearance of pulmonary metastasis in rat liver cancer (Sato, H.) were reported by other researchers. The most significant effects of SSM, however, have been the improvement of general symptoms in cancer bearing patients (disappearance of pain in particular), as seen in numerous reports on therapeutic examples and the prolongation of life. My interest in SSM was aroused in the following findings.

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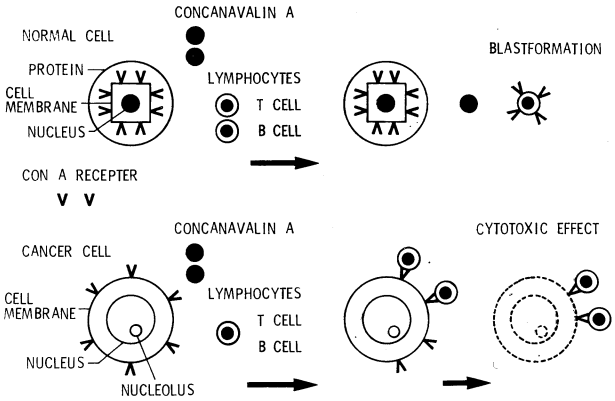

Fig. 1. Cytocidal effect of lymphocytes on cancer cells by cell to cell interaction.

While studying cancer cell membranes,[3] I noticed that a distinct cytotoxic effect of lymphotoxin was produced when cancer cells adhered to lymphocytes in the presence of concanavalin A *in vitro* (Fig. 1). In the course of study, I came across an autopsy finding of liver metastasis accompanying cervical cancer in a 47-year-old patient in whom lymphocytic cells swarmed and adhered to a part of the metastatic lesion causing impediment and disappearance of cancer cells. When I learned that this patient had been given SSM over a long period of time decided to investigate the antitumor activity of the substance. In the present study, in addition to clinical cases, experiments were carried out using more than 1,500 immune-deficient nude mice in which T-cells were defect. As a result, it was found that there was significant proliferation of collagen (collagenation) deriving from interstitium and mesenchyme distributed extensively throughout the body, and this collagenation acted as a biological mechanism against cancer. Details of the antitumor activity of proliferated stromal cells with SSM are reported here as a model.

Clinical Cases[4–8]

Below two cases of breast cancer selected from 10 clinical examples of patients in the terminal stage given SSM alone and their post-mortem findings are described.

Clinical case I: The patient was 43 years old when first examined in the Department of Surgery, Okayama Rosai Hospital, at which time she was found to have the left mammary cancer (adenocarcinoma).

The first medical examination revealed metastasis from the mammary cancer in the skin and the axillary lymph nodes. In addition, the arms could not be raised due to the formation of an ulcer in the breast skin resulting from cancer infiltration. Although a left oophorectomy was performed, it proved useless, since metastasis from the breast to both ovaries had already occurred and cancer infiltration was observed even in the pelvic cavity. Chemotherapy was also impossible due to general debility and anemia. Therefore, subcutaneous injection of SSM A (2 μg/ml, a polysaccharide) and SSM B (0.2 μg/ml) was carried

out on alternate days. Four months after the initiation of SSM therapy, infiltration foci and the ulcer in the skin diminished and the patient could raise her arms. A year later, the cancerous ulcer had disappeared completely, although it left a reduced lump. While metastasis in the lumbar area was observed during this time, the patient turned to work 6 months after treatment and continued

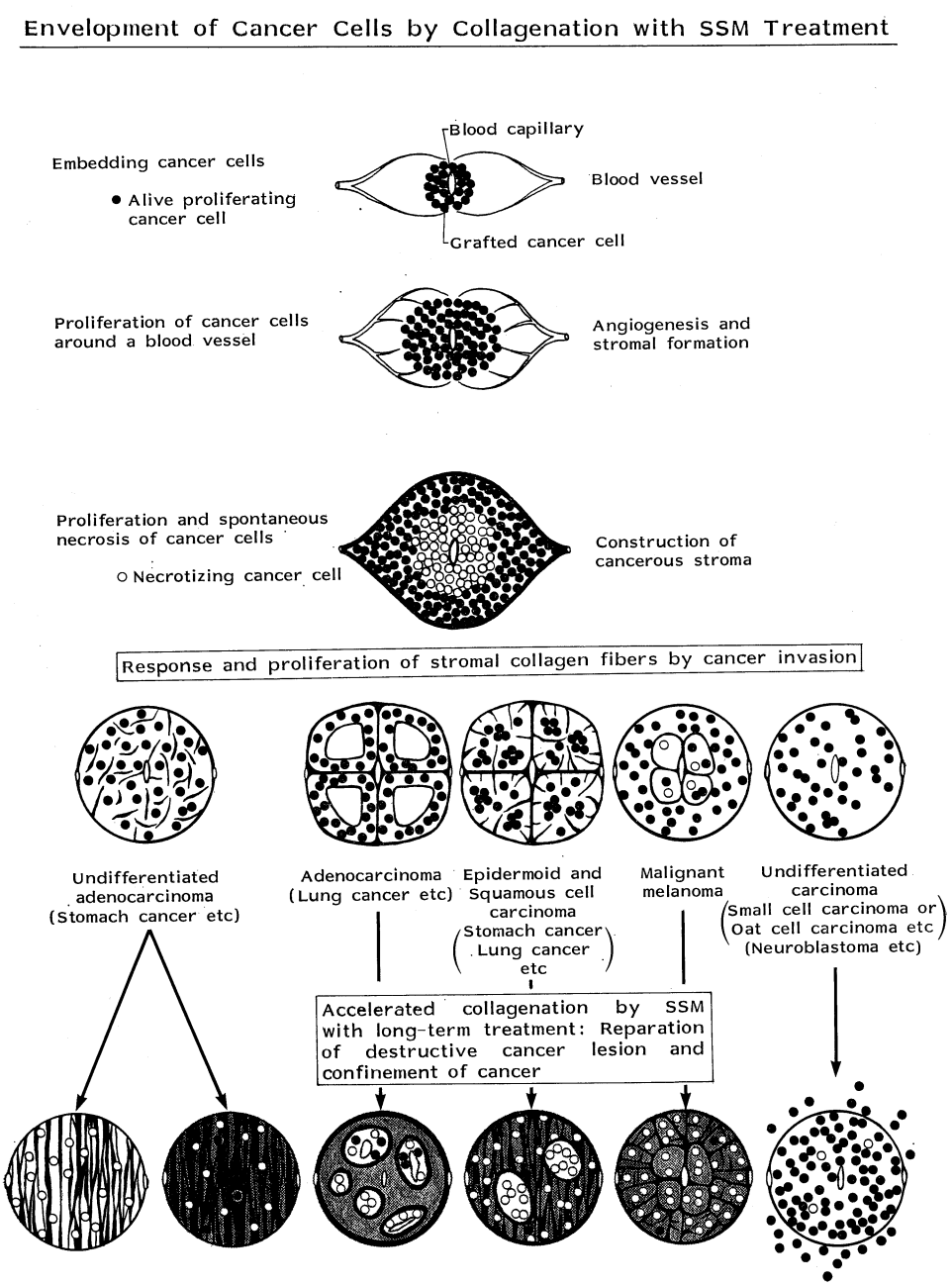

Fig. 2. Schema of collagen and stromal proliferation by cancer and acceleration of collagenation with SSM long-term treatment.

to work for nearly 6 years. During the 5th year, however, a small lump the size of the little finger-tip was deciduated from the uterus and was found to contain cancer tissue, most of which was distinctly replaced by collagenation.[8] The patient died 1 year later from cancerous peritonitis caused by lumbar metastasis. No opportunity for post-mortem examination was available.

Clinical case II[8]: The patient was 27 years old when first examined in the Department of Surgery, Okayama Rosai Hospital at which time she was found to have the left mammary cancer (adenocarcinoma).

One year postsurgically, metastasis was found in the left supraclavicular lymph node and the skin, and a bilateral oophorectomy was performed. Thereafter, single treatment with SSM was continued for 4 years and 8 months. The patient carried out housework during this period and, although innumerable skin metastases developed, infiltrated cancer cells shrank, degenerated and deciduated due to collagen envelopment produced by subcutaneous injection of SSM. Larger infiltration foci also cicatrized accompanying cancer proliferation and deciduated to be replaced by new skin. This finding showed a recovery course that was the same as that in the case of cancer xenografts of human lung cancer (adenocarcinoma) in nude mice.[4–7] An autopsy revealed multiple serous metastases in both pleura and the colon transversum as well as metastases in the thoracic skin, II lumbar, pelvis and ribs. Cancerous foci in these metastases, however, were all small, showing degeneration and disappearance of cancer cells due to "envelopment" by the distinct collagenation from cancer interstitium, which resulted in suppression and interference with infiltration. In almost all cancerous foci in which cancer cells degenerated and became necrotic due to collagen envelopment, calcium deposition was seen distinctly. During the long course of the disease, the hydrothorax was treated with mitomycin injections followed by large doses of SSM.

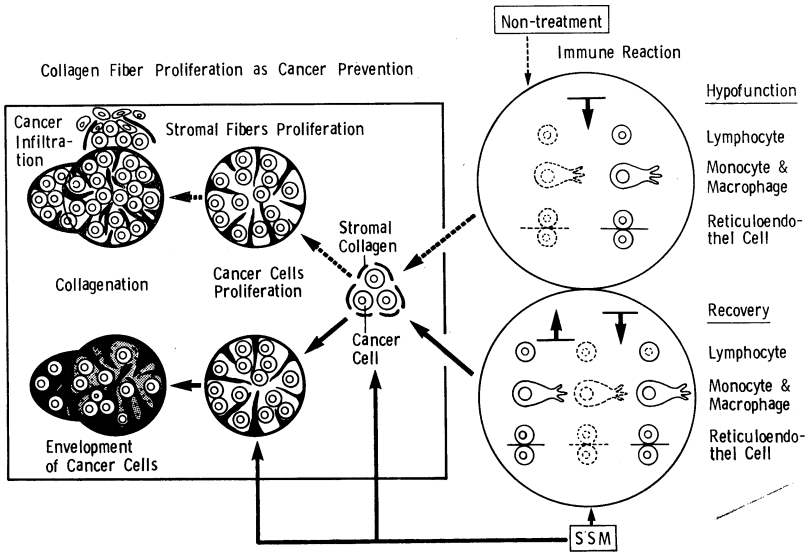

Fig. 3. Proposal of cancer preventive mechanism by SSM.

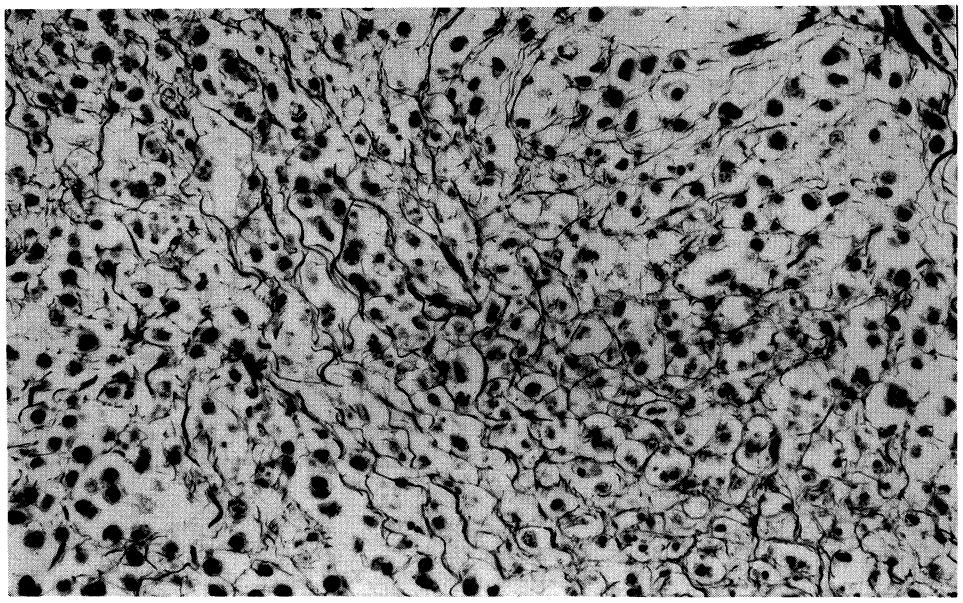

Fig. 4. HGC xenograft (2.5×10^7 cells) in a nude mouse. Collagen fiber production in the tumor. 86 days after transplantation and treatment with SSM 100 μg every other day.

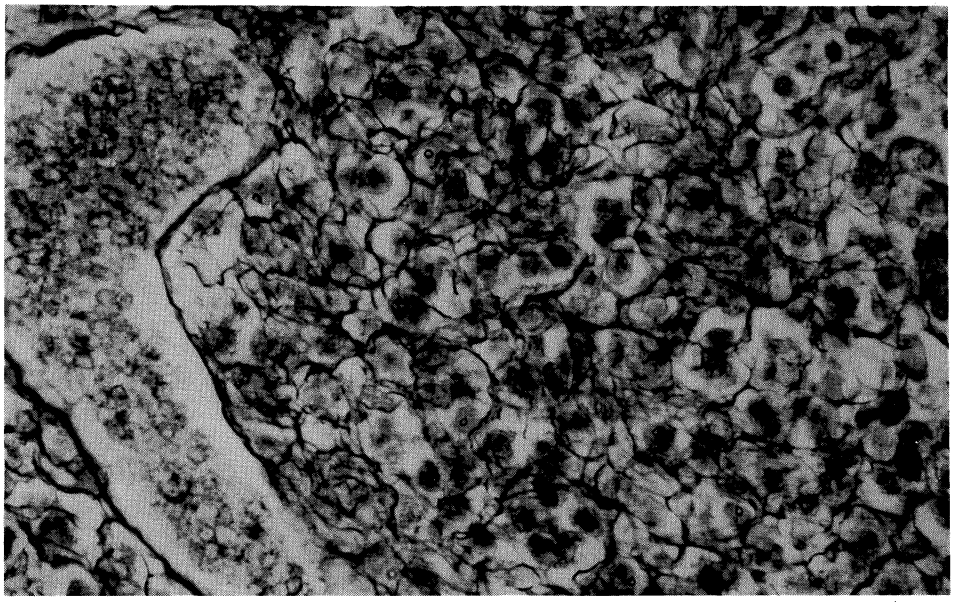

Fig. 5. HGC xenograft in a nude mouse. Remarkable proliferation of collagen fibers from the vascular wall against infiltrating cancer cells was observed 67 days after transplantation and treatment with SSM 100-200 μg every other day.

Study of various cultured cancer cells transplanted into athymic nude mice

Cancer interstitial reactions and collagenation were observed in the cancers of xenografts and allografts of various cancer cells transplanted into nude mice. However, histological findings did not reveal suppression of cancer proliferation

based on the mechanism of immune reaction by activating lymphocytes and macrophages by SSM treatment. Nude mice were used as an experimental model as T-cell participation could be completely eliminated and observation of human cancer cell proliferation by xenografts was possible in them. While numerous human cancer cells were used as grafts as can be seen in Fig. 2, mainly the cases of HGC (human gastric cancer, undifferentiated) (Figs. 4-6) and HLC (human lung cancer, adenocarcinoma) (Figs. 7-11), are described here. These cells show a strong vital implantation ability. The former was implanted completely with 1×10^7 cells and the latter with 3×10^6, and they

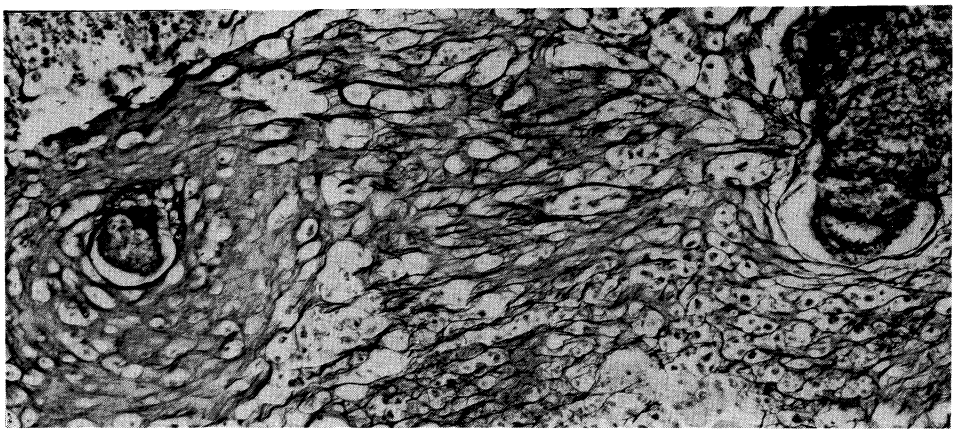

Fig. 6. Cicatrization by collagenation of HGC tumor. 86 days after treatment with SSM 100 μg every other day. Pap stain, $\times$100.

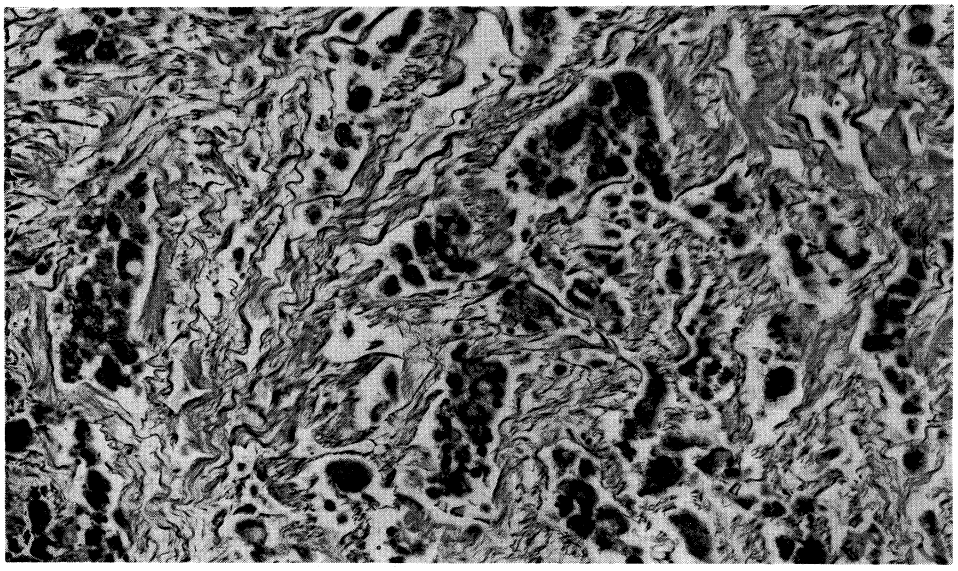

Fig. 7. HLC xenograft (6×10^6) of a nude mouse. Tubular pattern of collagen fiber proliferation and the tumor was cicatrized with treatment of SSM 100 μg every other day. 40 days after transplantation and SSM treatment. Pap stain, $\times$200.

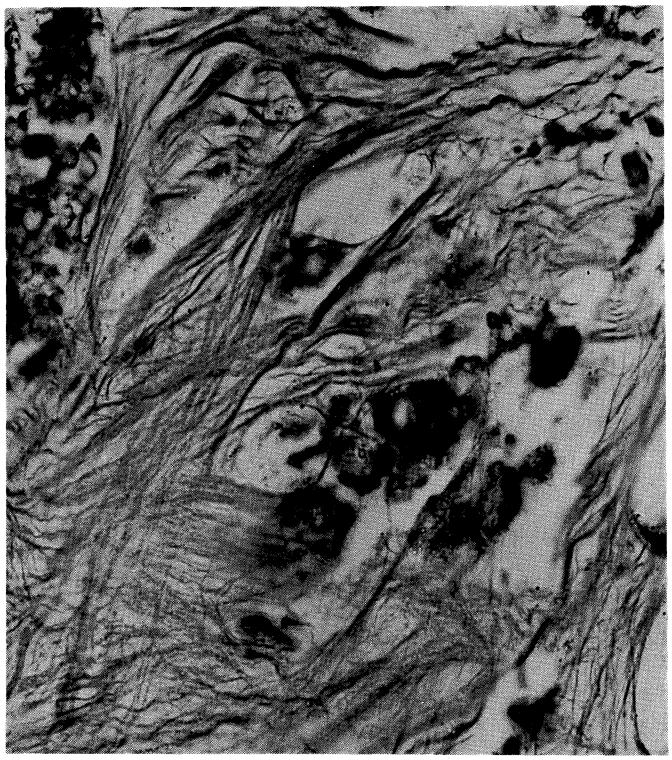

Fig. 8. Disappearing cancer cells due to remarkable proliferation of collagen fibers in the HLC tumor. 40 days after transplantation (6×10^6) and treatment of SSM 100 μg every other day.

both grew into chicken-egg size lumps 4 months later. Apart from these an NB41A3 (neuroblastoma, mice) was completely implanted with $3\sim6\times10^6$ cells, a malignant melanoma of human derivation G351, with 1×10^7 cells, and two of mice derivation, B-16 and Clone-M-3, respectively, with more than 3×10^6 cells, they later all exceeded chicken-egg size. Epidermoid carcinoma of human derivation, squamous cell carcinoma (pulmonary cancer PC-9, stomach cancer MKN-1) and oat cell cancer (pulmonary cancer PC-3) were implanted with 10^7 cells and grew.

Construction of cancer interstitium and angiogenesis in transplanted human cancer cells in nude mice

When cultured cancer cells were transplanted into nude mice, as can be seen in Fig. 2, blood vessels extended from the vicinity of the implantation site or from a distant place towards the cancer lesion and proliferation of fibroblasts could be seen 7 days later. The blood vessels extended into the existing interstitium surrounding the lesion and formed an interstitium with a double structure. Within 24-48 hours of transplantation, a small number of lymphocytes and macrophages in the flowing blood appeared temporarily and angiogenesis was seen in the inner layer of this interstitium 7 days later. In the original

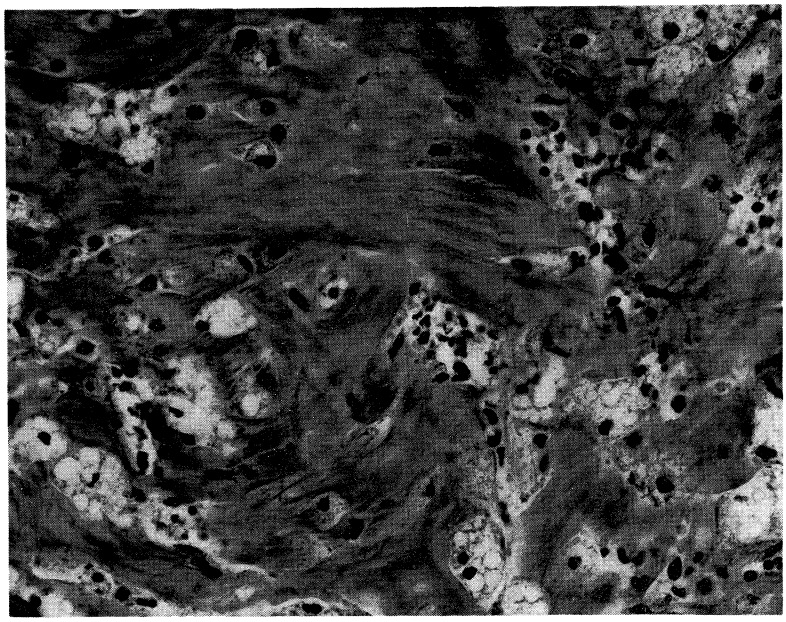

Fig. 9. HLC xenograft (6×10^6) of a nude mouse. The lobular proliferating cancer cells degraded (vacuolation) due to confinement by collagenation. 40 days after treatment with SSM 100 μg every other day. Mallory stain, $\times$400.

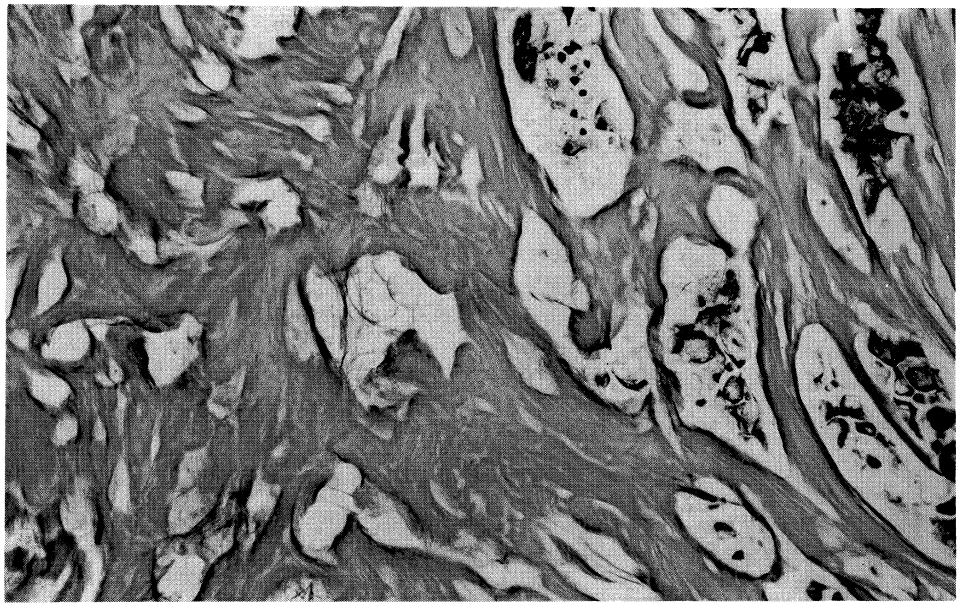

Fig. 10. HLC xenograft (6×10^6) of a nude mouse. Cancer cells disappeared due to confinement by remarkable proliferation of collagen fibers. 45 days after treatment with SSM 100 μg every other day at the same time as cell transplantation. Pap stain, $\times$200.

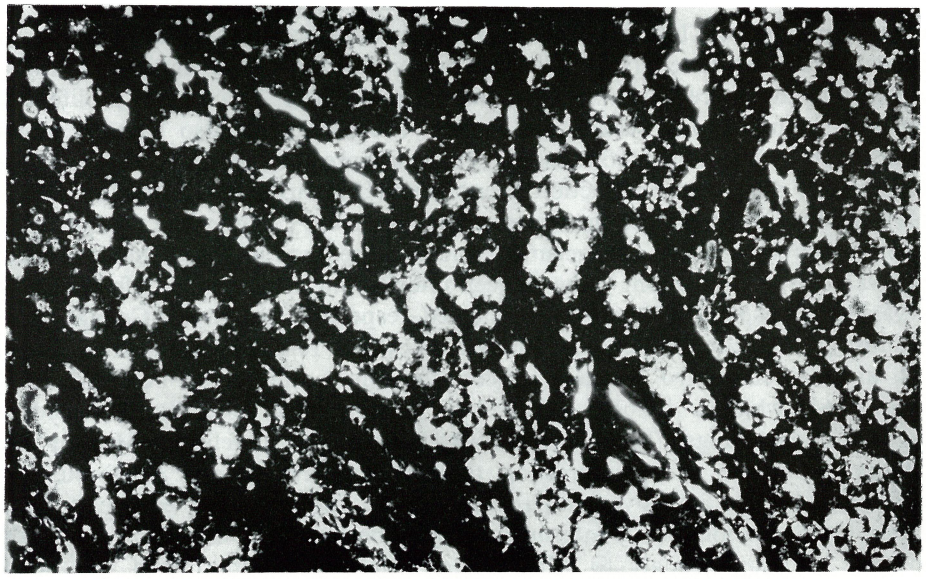

Fig. 11. Necrotizing and disappearing HLC cancer cells in the cicatrized tumor. 150 days after treatment of SSM 100 μg every other day.

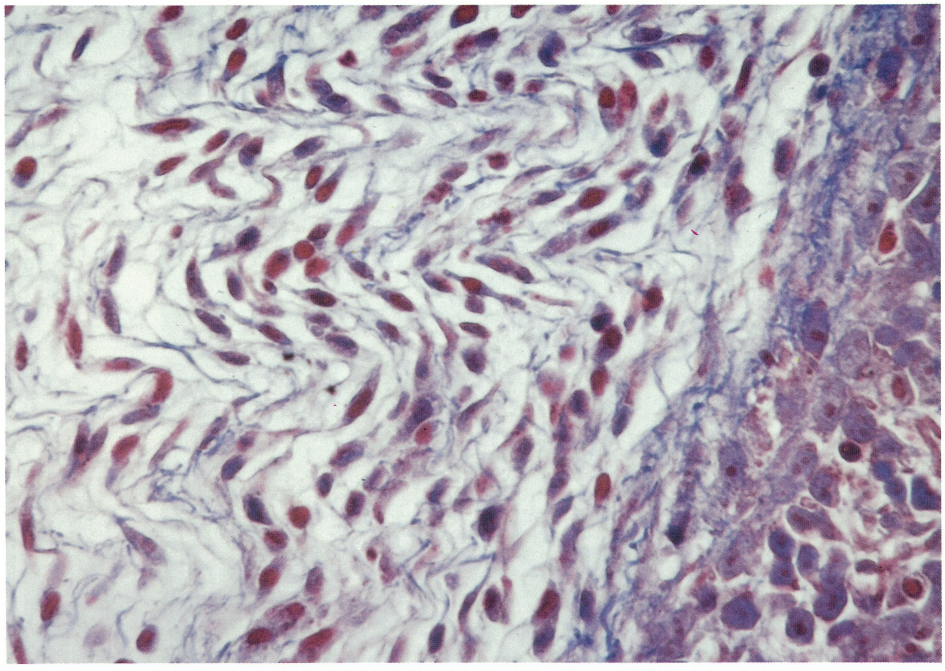

Fig. 12. The proliferation of fibroblastic cells derived from blood vessel of angiogenesis surrounding HGC tumor, which inbedded in a nude mouse. 5 days after transplantation. Lymphocytes were not found. Mallory stain, $\times$200.

interstitium, an outer layer of fibroblasts and reticular fibers (including macrophages) was constructed (Fig. 12) and newly formed blood capillaries invade further into the cancer lesion. More than 4 weeks later, cancer cells proliferated markedly around blood vessels and natural necrosis could also be seen in the circumferential lesion of blood vessels. Necrosis spread to central lesion by tumor proliferation due to reconstruction phenomena (Umbau). In the necrotic lesion, debris of the interstitial components could be seen. While the tumor grew larger in untreated mice, collagenation could be seen as in Fig. 2 in accordance with vascular construction. The cancer interstitium was not a uniform entity, as various transplanted tumor cells showed respective patterns. Collagenation sprouted in the circumference of individual cells in relatively diffused in cancers such as undifferentiated stomach cancer, showing a diffuse or boxing pattern (Figs. 4–6), while a tubular or alveolar pattern was generally indicated in adenocarcinoma (Figs. 7–11), with fibers reacting positively to Masson and Mallory staining. It is interesting that, in epidermoid and squamous cell carcinoma, boxing and alveolar patterns existed together, and extremely prosperous collagenation of an intermediate type between the diffuse and alveolar patterns, reacting strongly to Masson and Mallory staining from the initial

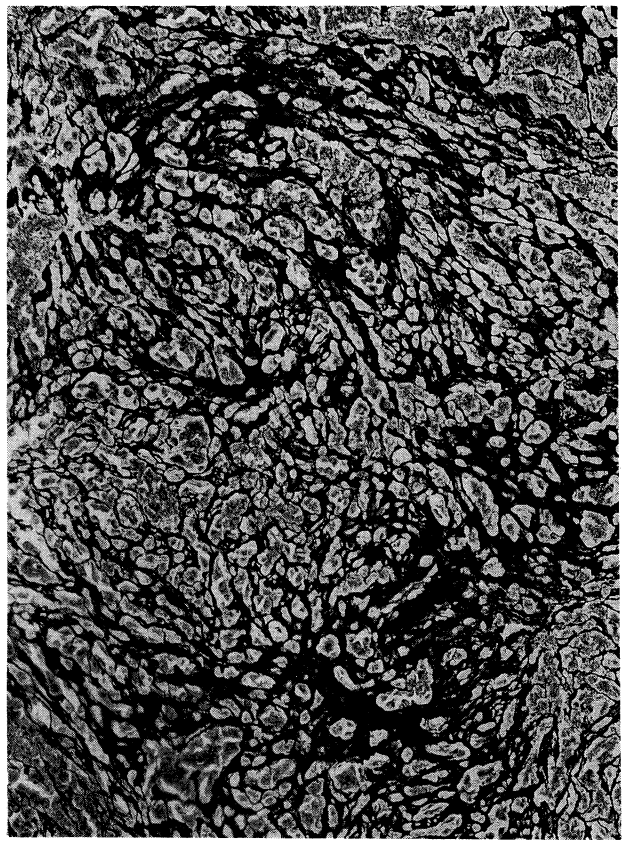

Fig. 13. Collagenation of an intermediate type between the diffuse and alveolar pattern of differentiated adenocarcinoma or epidermoid carcinoma (PC-9 : lung cancer) after 68 days treatment of SSM 40–50 μg every other day. Pap stain, $\times 200$.

stage of transplantation, could be seen (Fig. 13). On the other hand, in malignant melanoma of both human and mice derivation, collagenation of positive Mallory staining was weak even after one month even though silver impregnated reticular fibers, which are positive to Pap staining could be observed in ribbon forms

Fig. 14. Reticular fiber or ribbon formation in the xenograft grafted human malignant melanoma cells (G361, 10^7). 173 days after transplantation. Non-treatment control mice (♀). Pap stain, ×200.

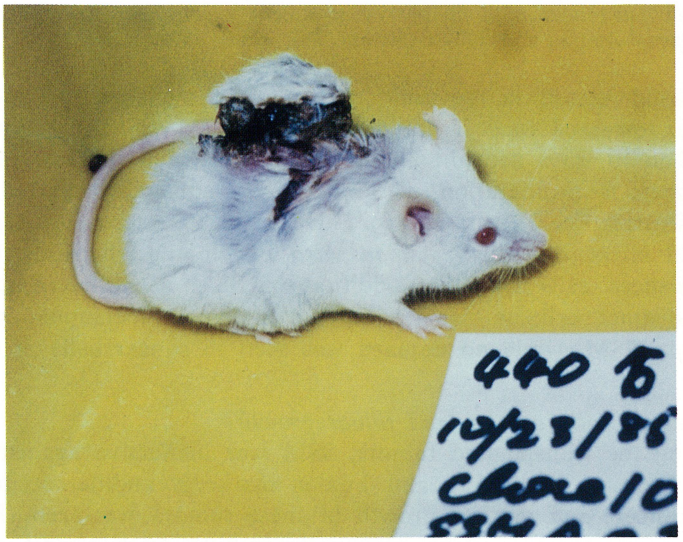

Fig. 15. Tumor grafted murine malignant melanoma cells (Clone-M-3: 10^7 cells, thymic Balb/c mice) were cicatrized and healed after 106 days of transplantation and subcutaneous injection of SSM 1 μg every other day.

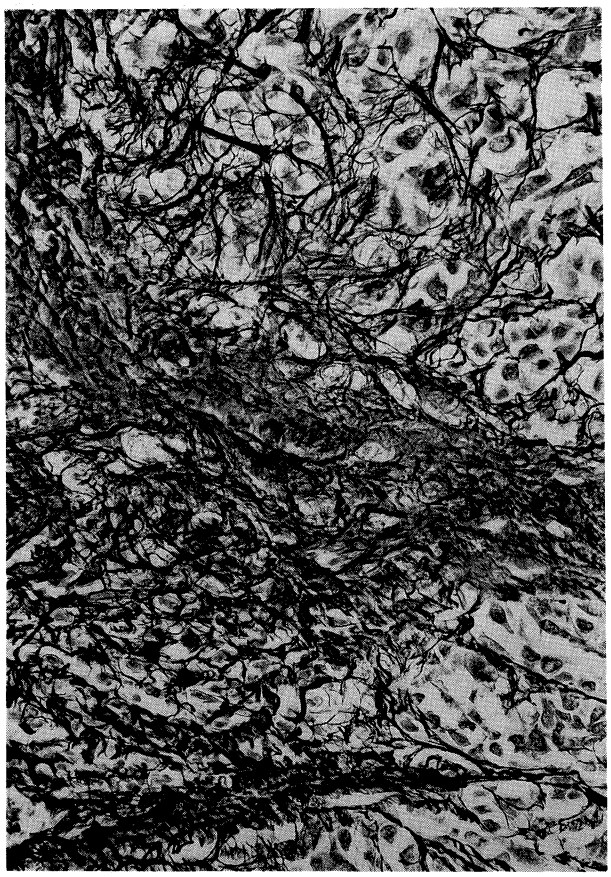

Fig. 16. Acceleration of collagen proliferation in the xenograft transplanted G361 cell (10^7) with SSM 50-100 μg treatment every other day. 87 days after transplantation and SSM treatment. Pap stain, ×400.

around blood vessels in the tumor center. Although these Pap-positive collagenations remained weak if not treated with SSM, SSM injection for a period of more than 40 days brought about degeneration and necrosis of tumor cells, since prosperous proliferation of Pap-positive collagen occurred resulting in intense cicatrization (Figs. 14-16). Here, it is interesting that SSM had a cytocidal effect on cells. An *in vitro* of cloning efficiency using various cultured cancer cells with the addition of SSM at different concentrations revealed distinct cellular damage only in malignant melanoma at a dose of 100-200 μg SSM, while no damage was seen in cancer cells of other types.

Significance of collagenation and tumor growth

Among the transplanted tumors, SSM was ineffective against proliferation of neuroblastoma (NB41A3) and human markedly undifferentiated carcinoma (oat cell cancer) and marked growth of these tumors was seen (Fig. 17). In these cases, vascular reaction and collagenation were almost or completely absent and the tumors eventually exceeded chicken egg size. Thus, it can be concluded that collagenation in the cancer interstitium participates greatly in

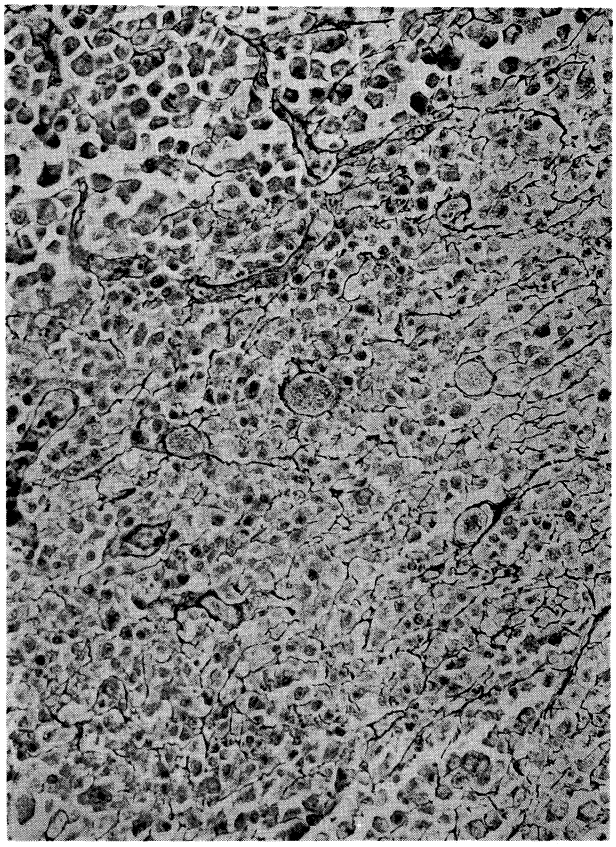

Fig. 17. No response of collagenation from the blood vessels was observed in the undifferentiated cancer cells (PC-6: oat cell lung cancer 2×10^7) xenograft and tumor grew to the size of a hen's egg. 52 days after transplantation.

suppression of tumor growth.

Change in cell membrane and collagenation[5,7]

As stated above, the initial construction of cancer interstitium around blood vessels and the collagenation following it seem to depend on glycoproteins such as fibronectin present in cancer cells, especially in their surface layer.

Here, it is significant that, while extremely undifferentiated small round cells such as NB41A3 and lung cancer or a transplanted cancer such as oat cell cancer grew around blood vessels, eventually exceeding chicken egg size, vascular reaction, such as the formation of reticular fibers from blood vessels, was completely absent,[7] allowing rapid tumor growth. Against these tumors SSM was ineffective. Therefore, I conducted the following experiment to determine why vascular fiber formation did not occur.

Tumor cells fixed in 0.25% glutaraldehyde, an electron microscopic fixative known to be the best for keeping the cell structure as close as possible to living conditions, and 3×10^7 cells were implanted subcutaneously in nude mice. Although vascular reaction was absent with living cells of the same cancer,

reticular fibers appeared and proliferated encircling tumor cells were observed in one week.[5,7] A small number of lymphocytes and macrophages appeared temporarily around cancer cells in 24-28 hours, but these cells did not act as immune controlling cells handling cytotoxic effect on tumor cells. On the other hand, collagenation from blood vessels was distinct, indicating its importance in relation to cytocidal effect on tumor cells. Therefore, I observed the structural changes caused by fixation with typical cell surface substances such a mannose, glucose, sialic acid, poly-L-lysine, N-acetyl-galactosamine, N-acetyl-glucosamine, N-acetyl-lactosamine and methyl-B-D-mannopyraminoside isoproplyate by the circular dichroic spectrum. The results showed that the glycoprotein of fibronectin alone brought about a structural change.

Collagen synthesis in a co-culture of interstitial and cancer cells

When the ability to synthesize collagen was examined using [^{3}H]-hydroxyproline radioactivity in a co-culture of cancer cells placed on top of a single layer of fibroblasts *in vitro*, collagen biosynthesis was observed biochemically in 24 hours. Fourteen days later, as shown by Pap staining collagen occupied almost all areas of the culture plate and cancer cells degenerated and deciduated. It is significant that collagen synthesis and its promotion were seen to occur by direct contact with interstitial cells and cancer in the absence of mediated cells such as immune cells (Fig. 18). It therefore can easily be imagined that a similar phenomenon occurs within living cancer bearing individuals. In addition, it must be noted that epithelium derived cancers such as those of the lung and stomach also possess prosperous collagen synthesizing ability as fibroblasts.[9-11]

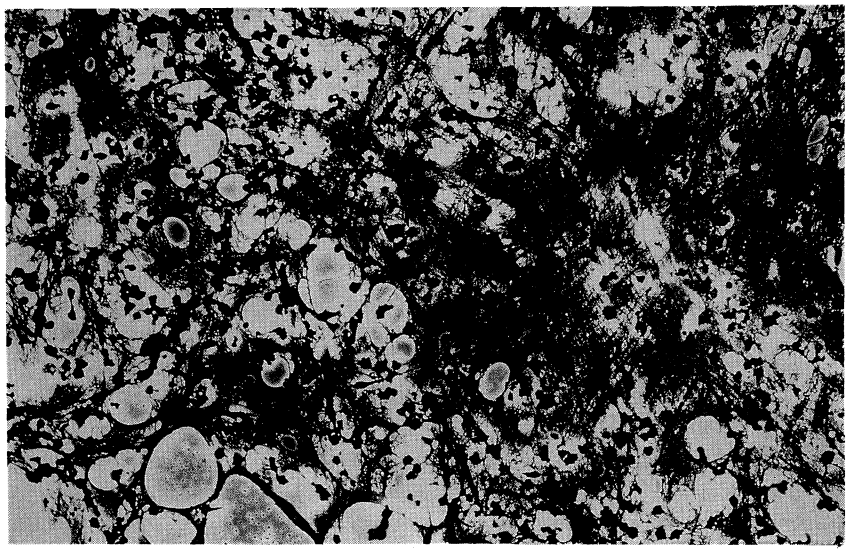

Fig. 18. Reticulum fiber (Reticulin) production in a co-culture of Detroid 550 fibroblasts and HGC. 4 weeks culture. Pap stain, ×400.

DISCUSSION

As cancer develops in a host, it comes across interstitial cells with widely distributed local blood vessels at their centers, or proliferation of fibroblasts as well as endothelial cells inevitably occurs due to angiogenesis. Although collagen is always biosynthesized as in *in vitro* experiments,[9–11] the intensity of collagenation becomes important because of the strong destructive invasion of cancer cells in many cases or, occasionally, because of the enzymes (collagenase) secreted by them. Then, the necessity of a direct cytotoxic combination of radiation and chemotherapies must be considered to control cancer proliferation. However, cancer cells themselves are often seen to fall into extensive and natural necrosis in the process of invasion. Long-term administration of SSM will normalize the lowered activity of lymphocytes, accelerate reparation of the lesion with interstitial components as materials and promote cicatrization of cancer lesion by all the collagen producing cells. As a result, "envelopment" (Figs. 3–6 and Figs. 7–11) of cancer cells by interstitial collagenation will occur in various forms, causing suppression of cancer infiltration and proliferation. Although the ideal cure of a transplanted cancer in nude mice is its complete disappearance, improvement is obtained when cancer growth stops or, when, while the cancer is still growing, collagenation progresses, causing cicatrization. Recently, Delinassios, J.G.[12] has also pointed out the significance of interstitial cells acting as a tumor degenerating factor (TDF).[12]

Although cancers possess different characteristics and exhibit individuality in their patterns of constructing cancer interstitium as can be seen in Fig. 2.

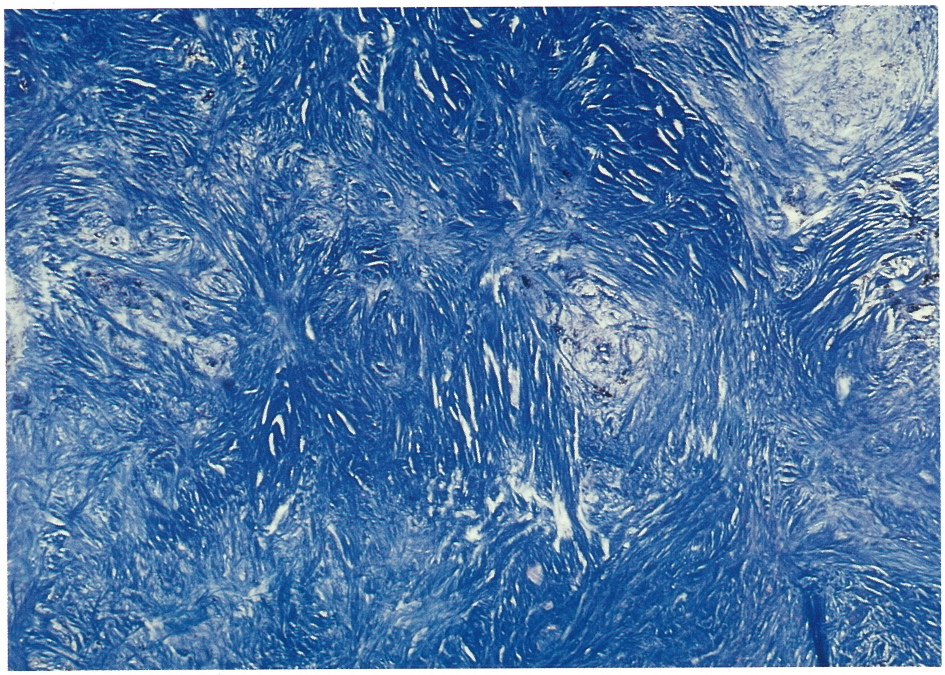

Fig. 19. Pulmonary tuberculosis healed by remarkable collagenation. 69-year-old man. Masson stain, ×40.

It is significant that the majority of cancers form interstitium, though different in extent. As participation of SSM in collagenation is considered, it is the reason why the substance becomes widely useful in anticancer effect through collagenation (Fig. 3). In a similar manner to recovery from tuberculosis progresses by fibrosis (Fig. 19) and cicatrization, with collagenous fibers surrounding the residual blood vessels left in a caseous lesion, cancer infiltrated lesions are reconstructed by long-term injection of SSM and "envelopment" of cancer occurs through advancement and acceleration of collagenation. This fact suggests tuberculosis and cancer are removed phenomena but can be said as extremely resembling pathological findings in the aspect of reparative mechanism. Throughout the pre- and early post-war periods, tuberculosis was so widespread in this country that it was said to be a national disease and many young lives were regrettably lost. Revolving this abominable *Mycobacterium tuberculosis*, excellent researchers were published and, particularly, Y. Yamamura,[13] the former president of Osaka University, conducted an international study concerning the experimental cavity formation due to allergy using proteolipid. At present, polysaccharide components extracted from human-type *Mycobacterium tuberculosis* are known to interfere with the proliferation, infiltration and metastasis of cancer by collagenation in a similar manner they promote cure from tuberculosis and these phenomena can be considered as "light and shadow" concerning studies in *Mycobacterium tuberculosis*.

I add that a DNA nucleic acid[14,15] extracted from *Mycobacterium bovis* BCG has been shown recently to have a cytocidal effect on cancer and this has gathered much attention.

REFERENCES

1) Maruyama, C.: On the treatment of malignant tumors with an extract from tubercle bacilli (Maruyama Vaccine) with the summary and some illustrations of the clinical results in 1965-1971. Research Institute of Vaccine Therapy for Tumors and Infectious Diseases. pp. 1-68. Nippon Med. Univ., Tokyo, 1973
2) Hayashi, Y., Ebina, T., Suzuki, F. and Ishida, N.: Interferon inducing activity of an immunotherapeutic anticancer agent SSM prepared from Mycobacterium tuberculosis strain Aoyama B. Microbiol. Immunol. **25** : 305-316, 1981
3) Kimoto, T., Ueki, A. and Nishitani, K.: Phagocytosis of lymphoblastoid cells and cell destruction of human malignant tumor cells. Acta Pathol. Jpn. **25** : 99-114, 1975
4) Kimoto, T.: Collagen and stromal proliferation as preventive mechanisms against cancer invasion by purified polysaccharides from human tubercle bacillus (SSM). Cancer Detect. Prev. (U.S.A.: Alan R. Liss, Inc.) **5** : 301-314, 1982
5) Kimoto, T., Watanabe, S., Hyodoh, F. and Saito, T.: Collagen fiber formation and proliferation as a mechanism of cancer prevention and regression induced by extract from Mycobacterium tuberculosis: Correlation between clinical observation and animal experiments. Cancer Detect. Prev. (U.S.A.: Alan R. Liss, Inc.) 1988 (in press)
6) Kimoto, T.: Protective proliferation of collagen against cancer induced by polysaccharides of human-type Mycobacterium tuberculosis: Double xenograft study. Cancer Detect. Prev. (U.S.A.: Alan R. Liss, Inc.) 1988 (in press)
7) Kimoto, T. and Watanabe, S.: Antitumor effects of collagenation on human cancer by polysaccharides of human type Mycobacterium tuberculosis using the experimental models of xenografts. Acta Pathol. Jpn. **37** : 1743-1761, 1987
8) Kimoto, T.: Pathological studies on the three cases treated with polysaccharides of human-type Mycobacterium tuberculosis: Antitumor activity by collagen fiber proliferation. Acta Pathol. Jpn. **37** : 1919-1934, 1987
9) Sakakibara, K., Suzuki, T., Motoyama, T., Watanabe, H. and Nagai, Y.: Biosynthesis of an interstitial type of collagen by cloned human gastric carcinoma cells. Cancer Res. **42** : 2019-2029, 1982

10) Sakakibara, K., Takaoka, T., Katsuta, H., Umeda, M. and Tsukada, Y.: Collagen fiber formation as a common property of epithelial liver cell lines in culture. Exp. Cell Res. **111** : 63-71, 1978
11) Naito, Y., Kino, T., Horiuchi, K. and Fujimoto, D.: Promoting of collagen production by fibroblasts with gastric cancer cell in vitro. Virchows Arch. [B] (Cell Pathol.) **46** : 145-154, 1984
12) Delinassios, J.G.: Cytocidal effects of human fibroblasts in HeLa cells in vitro. Biol. Cell **59** : 69-78, 1987
13) Yamamura, Y., Kusunose, M. and Kusunose, E.: Lactic oxidase of Mycobacterium tuberculosis avium. J. Biochem. **39** : 227-238, 1952, Nature **170** : 207-208, 1952
14) Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fukuda, T., Fujisawa, Y., Furutani, Y., Yano, O., Kataoka, T., Sudo, T., Makiguchi, N. and Suganuma, T.: Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis ECG. I. Isolation, physicochemical characterization, and antitumor activity. JNCI **72** : 955-962, 1984
15) Shimada, S., Yano, O., Inoue, H., Kuramoto, E., Fukuda, T., Yamamoto, H., Kataoka, T. and Tokunaga, T.: Antitumor activity of the DNA fraction from Mycobacterium bovis BCG. II. Effects on various syngenic mouse tumors. JNCI **74** : 681-688, 1985