Review

Patho-Biological Observation on Biological Response Modifier (Maruyama Vaccine) in Cancer : Important Roles of Collagen Fibers Proliferation as Prevention of Cancer Development and Metastasis

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Polysaccharides (arabinomannan and mannan) extracted from human Mycobacterium tuberculosis (Aoyama strain) (Specific Substance Maruyama : SSM and Z-100)

In the 1970s various types of cytokines were discovered.[1] As oncogenic immunology advanced, it was clarified that immunological response in a host is based on cell to cell interaction such as T, B cell lymphocytes, NK cells and macrophages. In addition, the manufacturing of monoclonal antibodies, which recognize only a single antigen of a tumor cell, became possible. In the 1980s, the application of biologically active substances, which are milder and have less side effects than chemotherapeutic anticancer drugs, was proposed. These substances, including cytokines, were referred to as biological response modifiers (BRM).[2] IFNs, interleukins, and lymphocyte activating factors (LAFs) were discovered,[3,4] and their application in the LAK treatment is now being clinically tested. Although many cytokines and CSFs (colony stimulating factors) have been discovered in recent years, most studies of a host's biological response to BRMs focus on the direct cytocidal effects of cytokine on tumor cells. The behavior of cancer proliferation which has been treated with BRM has not yet been pathologically studied. In the present study, therefore, based on pathological findings in cases who were mainly cured by arabinomannan extracted from human-type *Micobacterium tuberculosis* (SSM, Maruyama vaccine),[5] I would like to state my opinion about the viability of an alternative cancer treatment by cicatrization, and the behavior of collagen proliferation in cancerous lesions. I will pathologically discuss the biological preventive mechanism of a host against cancer, and the mechanism for prolonging survival life when a host is stricken with cancer. Until today, findings from the Maruyama vaccine studies show that fibrous components from muscle cells,

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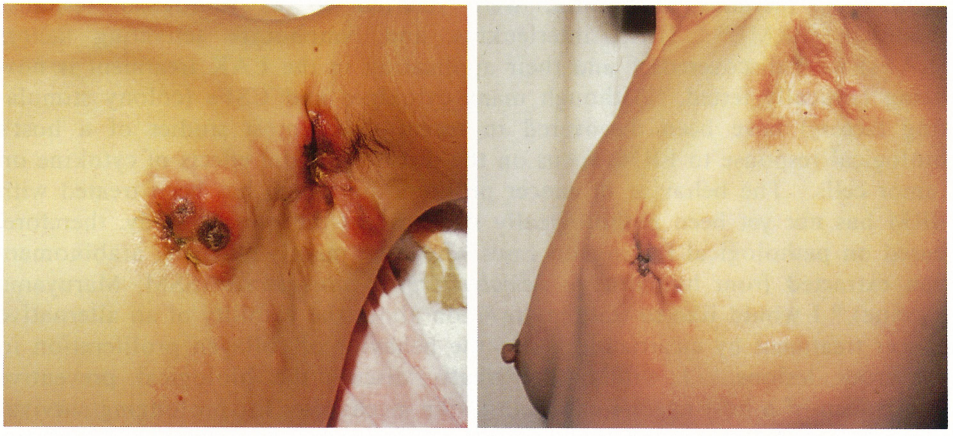
nerve cells and chondrocytes including stromal cells of cancer lesion as well as collagen fiber proliferation by cancer cells, play important roles in the biological defense mechanism treated with BRM. These findings are based on non-specific immunological phenomena (mainly caused by T-cells, macrophages and NK-cells). This biological preventive mechanism is similar to the mechanism used for curing tuberculosis, which is based on immunoreaction.[6-8] I will also discuss collagenation which is based on the character of tumor cells such as evidenced in breast cancer.[9] Collagenation is promoted secondarily by increased immunoreaction, due to accelerate T-cells, NK cells or macrophage activities.

Clinical cases of breast cancer which motivated us to begin the Maruyama vaccine study[9]

Before observing these two cases, the infiltration of lymphocytes and macrophages in cancer lesion was, in my opinion, the most important finding in immunotherapy; thus, I had concentrated solely on cytotoxicity. In these breast cancer cases, the infiltration of lymphocytes and macrophages was pathologically insignificant, while collagen proliferation of interstitium played an important role in the cure. To clarify the relationship between collagen proliferation and the character of tumor cells, I experimented on nude mice. In addition, the biological defense mechanism of BRM was studied on patho-biological significance of collagen fiber proliferation.

Case 1 (Surgery, Okayama Rosai Hospital)

A 43-year-old woman with left breast cancer (solid-tubular adenocarcinoma). At the first examination, metastasis to the skin and axillary lymph nodes was noted, and because of ulceration due to infiltration of cancer cells on the breast skin, she was unable to raise her hand (Fig. 1A). As the case was considered inoperable, left ovariectomy was initially performed. Since bilateral metastasis to the ovary and infiltration of cancer cells in the pelvic



A B

Fig. 1A. Clinical case 1. Okayama Rosai Hospital, Surgery. A 43-year-old female with left breast cancer (solid-tubular adenocarcinoma). Many metastatic foci and cancerous ulceration can be observed on the skin before SSM treatment.
Fig. 1B. Clinical case 1. Cancerous ulceration healed after 1 year of SSM treatment alone.

cavity were observed, SSM alone was administered (SSM-A and B, by subcutaneous injection every other day). Four months later, skin ulceration was reduced, facilitating biopsy, and allowing her to raise her hand. The histological findings at this time, revealed marked proliferation of collagen fibers. Growth of the basal membrane in ductal structures, argyrophilic fibers produced from small blood vessels. Individual cancer cells or cancerous lesions were surrounded by these fibers, as a result of which most of the cancer cells were atrophied and degenerated, showing marked inhibition. No infiltration of lymphocytes was noted in any of these lesions.

One year after injection of SSM-A (2.0 μg/ml polysaccharides contained arabinomannan) and B (0.2 μg/ml polysaccharides contained arabinomannan) the cancerous ulceration was cured, allowing free raising of the hand (Fig. 1B), and although the cancer remained for 6 years, no ulcer recurrence was noted. No opportunity for autopsy was available.

Case 2 (Surgery, Okayama Rosai Hospital)

A 31-year-old female. After an operation for left breast cancer (infiltrating solid-tubular adenocarcinoma), due to multiple metastasis, the patient was treated with SSM-A and B administered by injection over a long-term as an outpatient for 4 years and 8 months.

SSM-A and B were subcutaneously injected every other day for about 4 years until the time of her death. The clinical course was as follows:

October 27, 1982: Retention of left-sided cancerous pleural effusion relieved by massive intrathoracic injection of SSM (November 1, 1982 - January 8, 1983). Retention of right-sided cancerous pleural effusion relieved by massive intrathoracic injection of SSM. X-ray examination at the beginning of January, 1984, revealed metastasis to cervical vertebra C5.

January 6, 1984, readmitted. For 4 years after surgery, the course was not painful in spite of multiple metastasis, so that she was able to manage her

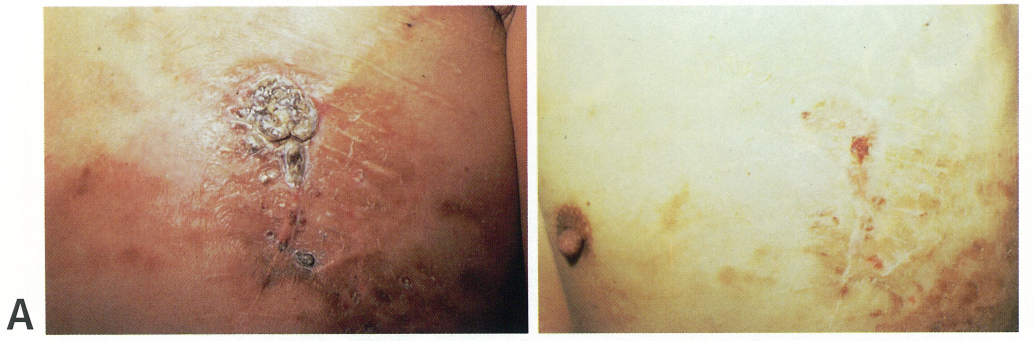


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Fig. 2A. Clinical case 2. Okayama Rosai Hospital, Surgery. A 30-year-old female. Left breast cancer (solid-tubular adenocarcinoma). A mastectomy had been carried out when metastasis to the axillary lymph nodes occurred 3 years previously. Treatment with SSM alone was performed. After the operation, the cancer continued to infiltrate diffusely into the skin and the other lymph nodes. About 4 years after the operation, the cutaneous lesion of the metastatic cancer formed cicatrices by treatment with SSM. This finding was similar to that in nude mice treated with SSM.

Fig. 2B. Clinical case 2. Female. Left breast cancer and metastasis. The cancerous cicatrices were shed and replaced by newly regenerated skin.

housework while being treated as an outpatient. During this period, as a result of massive subcutaneous SSM-A injection, the metastatic focus on the skin on the left side fell off (Fig. 2A,B).

Pathological findings

Every metastatic focus showed extremely sharp encapsulation, being small and localized in spite of long-term presence of cancer. No gross metastasis in the lung was found, and it was noteworthy histologically that metastatic foci were localized to the pleural interstitial tissue (in lymphatic vessels), neither lymphocyte activation nor cytotoxicity being noted. The major lesions were superficial metastasis to the pleura, renal capsule and cancerous peritonitis. Proliferation of cancer cells was remarkable at the lumbar vertebrae, with osteoclasia due to diffuse infiltration of undifferentiated adenocarcinoma. Even in this lesion, proliferation of collagen was remarkable, showing meshes surrounding every cancer cell. Small and localized metastatic foci were scattered in the liver (Fig. 3), the largest being as large as a thumb tip, and nearly all of them were smaller than red beans, with sharp encapsulation. Because of the marked collagenation, every cancerous focus showed fibrotic meshes, i. e., confinement due to proliferation of lattice fibers mainly consisting of vascular components and fibroblasts in the portal spaces.

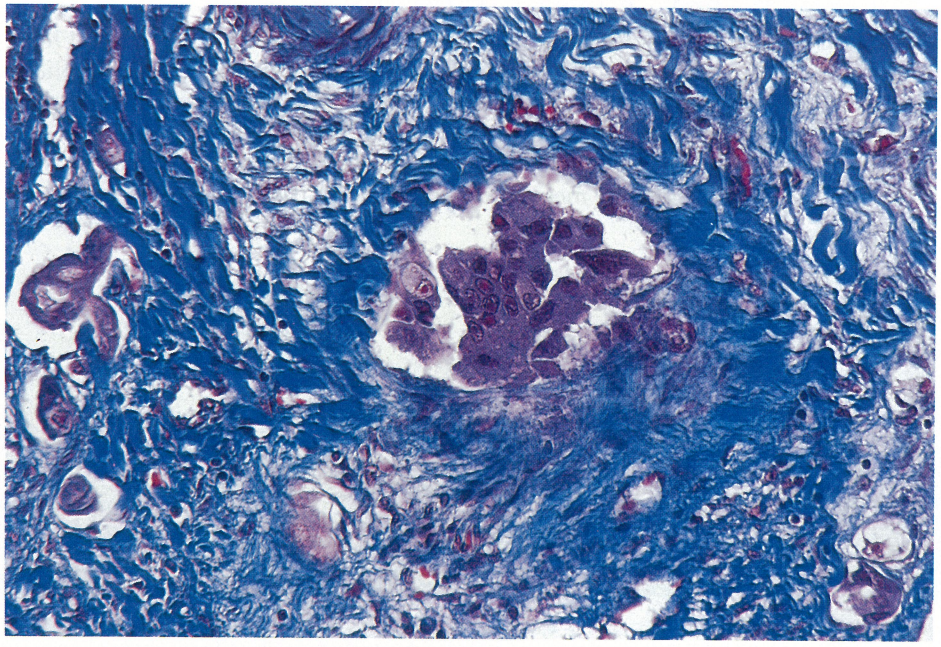


Fig. 3. Autopsy finding in clinical case 2. Disappearance of metastatic cancer cells confined by collagen fibers in the liver. Masson stain, ×200.

Stromal collagen fibers proliferation and new vascular formation around cancer tissue

Stromal proliferation in and around cancer cells is a common histological

observation. Such proliferation leads to the formation of cancerous stroma and fine vascular network in the tumor. Vascular architecture appears to be intimately related to cancer cells as Anoprunk *et al.* reported.[10] Furthermore, emerging evidence indicates that responses and proliferation of stromal collagen play an important role in preventing the development and metastasis of cancer. The mode of response and proliferation of stromal collagen vary, however, among cancer cell types. In some cases, collagen proliferates markedly and surrounds cancer cells, while in others on collagen proliferation can occur. Using SSM as a model, we have shown that collagenation is an important protective reaction against tumor growth in man.[6-9,11]

The antitumor effect of long-term use of SSM in patients with advanced cancer has been described.[9,12] We reported that long-term use of SSM facilitated the repair of destructive lesions induced by cancer invasion and results, in optimal cases, in cicatrization and cure. Histologically such lesions showed confinement and suppression of cancer cell proliferation (Fig. 4).

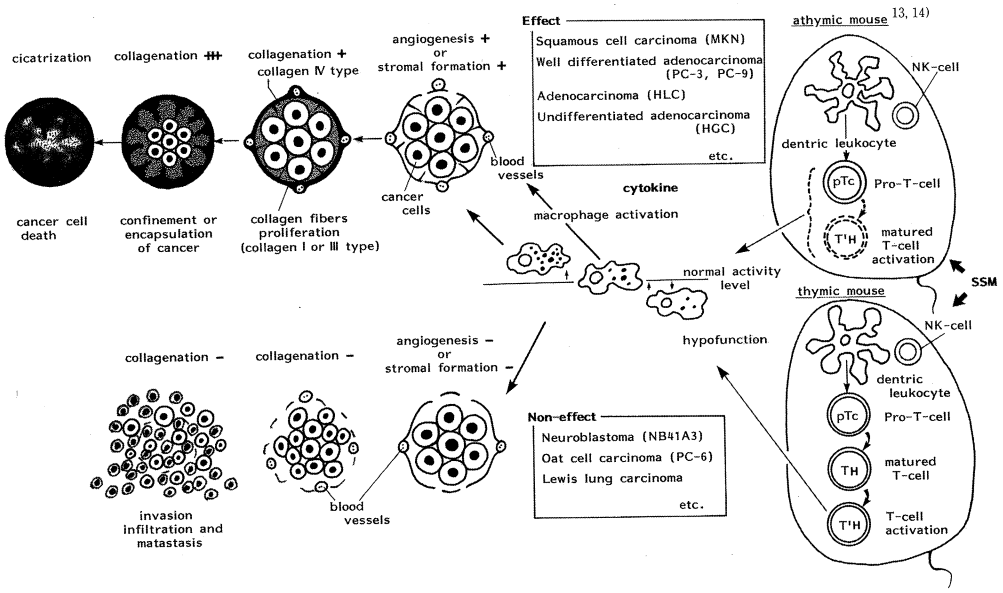


Fig. 4. Schema of encapsulation, confinement, and cicatrization of cancer by promoted proliferation of collagen fibers through macrophage activation with SSM and Z-100 long-term treatment.

Xenografts of human derived cancer cells to the athymic mice[6-8,11]

Although it is well-known that the SSM activates T-cells and macrophages, stimulates the production of IFNs,[15-17] and produces interleukin I in vitro, its most effective feature as an anti-cancer activity is that a high concentration (Z-100[18] 0.2 mg/cc) can be used over a long-term without side effects, and survival life significantly in coexistence with cancer.[19] However, experiments on animals about the cytotoxic or anti-tumor effects of SSM had not been successful for a long time, since the grafted tumors were sarcomas and observation periods were limited to 2-3 weeks. Cancer is a typical chronic

disease, which develops over a long time from its initial stage to death, so a duration of 2 to 3 weeks is in adequate. These studies did not clarify the behavior of cancer from a clinical viewpoint. Based on the clinical findings of these cases with breast cancer, we studied the interstitial proliferation, which is based not only on immunology but also the molecular structure of cancer cell

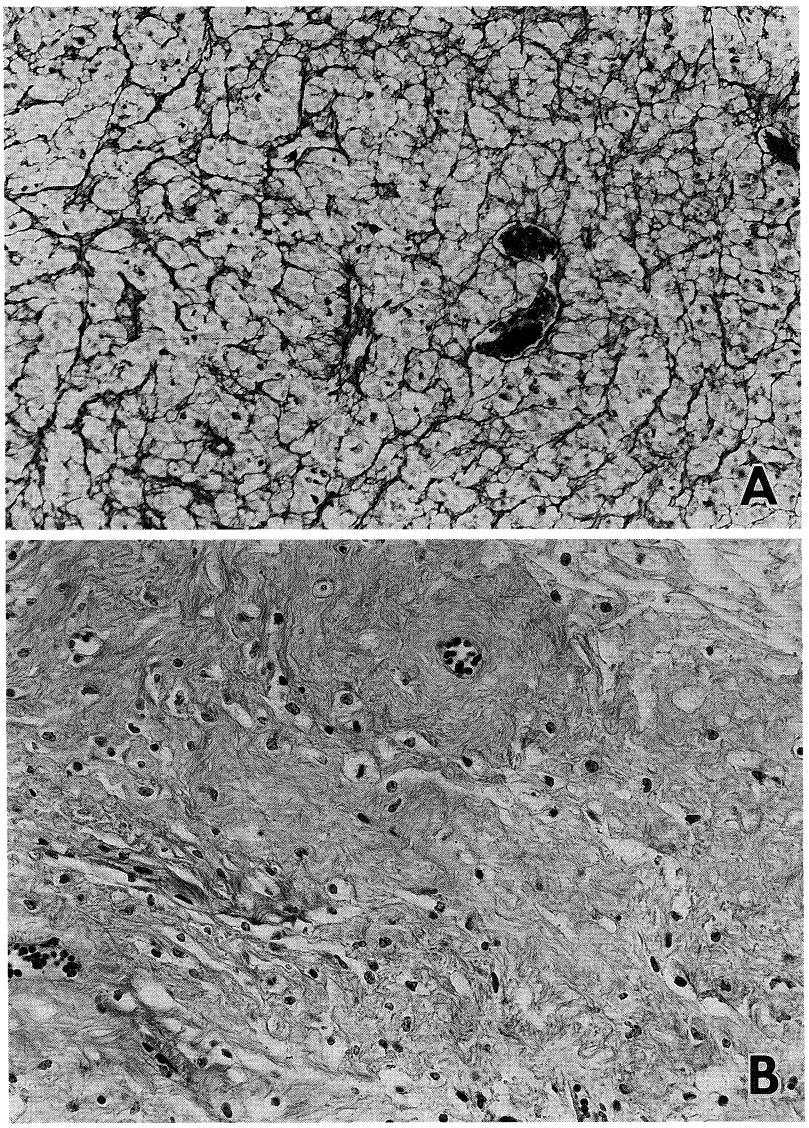


Fig. 5. Xenografts of HGC cells.
A. Nude 151-3. HGC cells (2×10^7) transplantation. Control. 23 days. Pap silver stain, $\times100$.
B. Nude 151-3 Nodular cicatrization of HGC tumor by collagenation. 86 days after treatment with Z-100 (100 μg) every other day. Pap silver stain, $\times100$.

membranes, and stromal reaction.[20] We also studied cicatrization, which may be caused by increased collagen fiber proliferation in clinical cases.[5,9] We used human cancer cells, which had been grafted onto athymic and thymic mice.[21] Collagen proliferation plays an important role in the biological defense mechanism of a cancer bearing animals and SSM stimulates the confinement, encapsulation, cicatrization of the cancer lesion, and inhibits metastasis.

Cultured cancer cells grafting to nude mice

Human gastric cancer cells: HGC (undifferentiated),[6-8] TRMKN-1 (squamous carcinoma),[22] TRKATO-III (signet ring cell carcinoma), and human lung cancer cells: HLC (adenocarcinoma),[6-8] TRPC-6 (small cell or oat cell carcinoma),[22] TRPC-9 (well differentiated adenocarcinoma, epidermal carcinoma)[22] were used. TRMKN-1, TRPC-3, TRPC-6 and TRPC-9 were prepared by reculturing nude mouse xenografts of MKN-1, PC-6 and PC-9, respectively, all of them purchased from Immuno-Biological Lab (Tokyo, Japan). These cancer cells were cultured in MEM supplemented with 10% FBS under humidified 5% CO_2. The xenografts (10^7 cells transplantation) prepared from the cancer cell line were histologically examined with regard to collagen proliferation and used for therapeutic study. The rate of successful transplantation for KATO-III was 0/10. Murine Lewis lung cancer cells, which had 100% success in grafting into athymic and C57/black mice,[22] were also used.

Nude mice were bred as aseptically as possible (ATCL-4 Clean Rack, Nippon Clea).

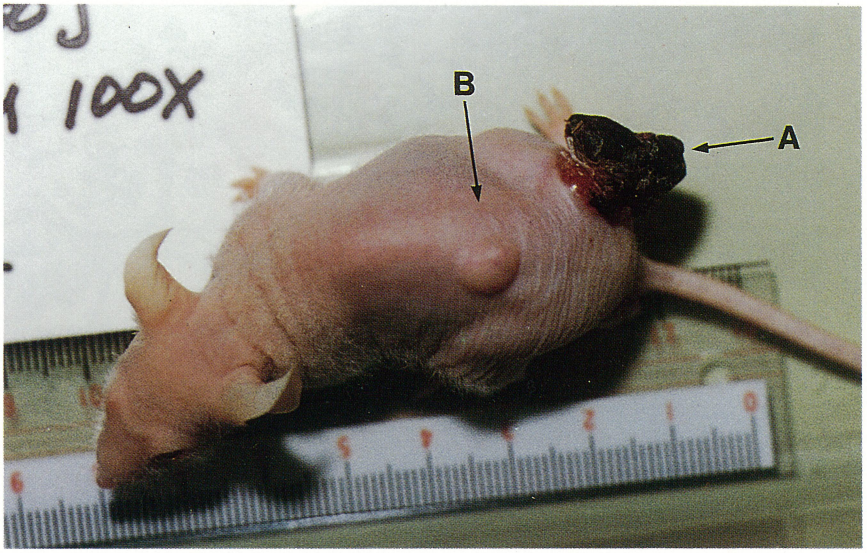


Fig. 6. Double xenografts of HLC cells (6×10^6) and HGC cells (1×10^7) were made in a same nude mouse. The tumor shows remarkable collagenation and cicatrization of the HLC tumor (A). The HGC tumor (B) shows inhibition of growth of the tumor 58 days after transplantation and treatment with Z-100 (50 μg). Different collagenation patterns in tumors were observed depending on characteristics of different individual cancer cells.

Subcutaneous and intra-tumomral injections of SSM

SSM A and Z-100 (1-100 μg) was subcutaneously (s.c.) injected at locus remote from the grafted cancer or directly into the tumor (i.t.) simultaneously

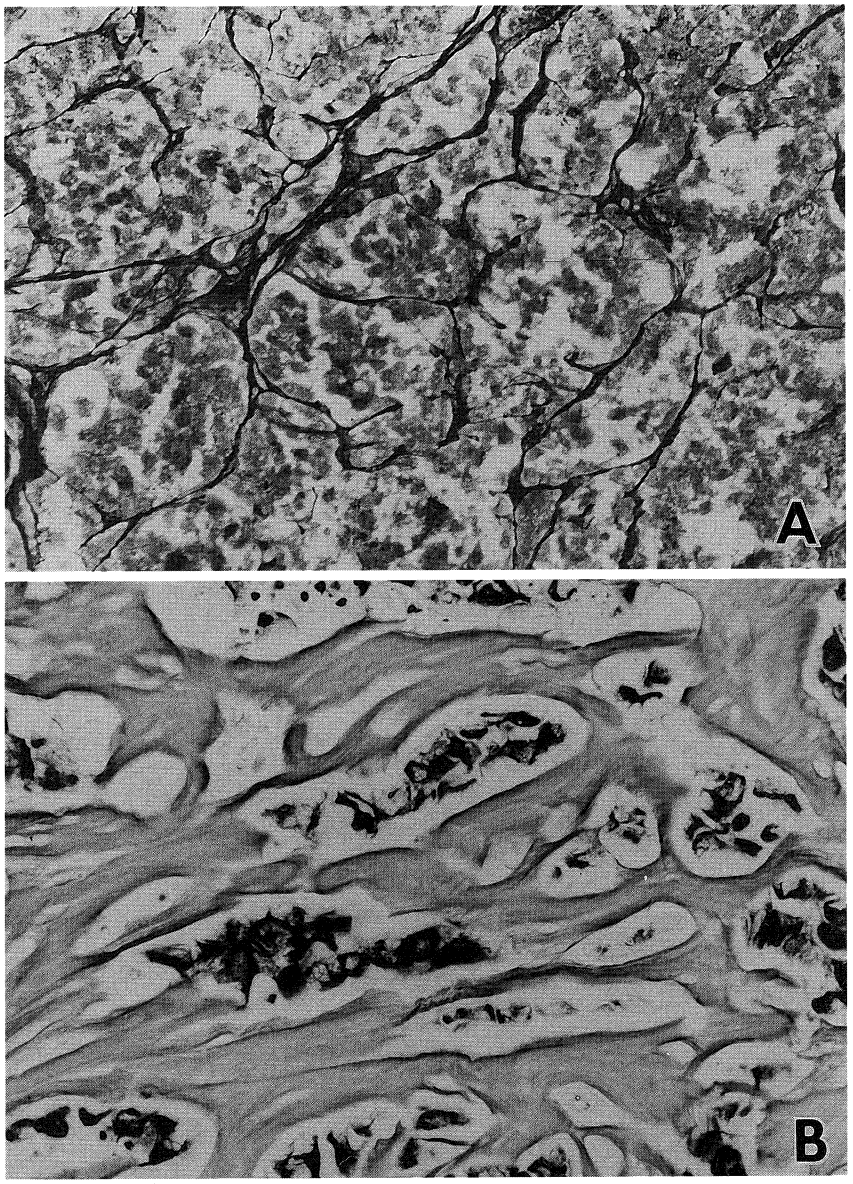


Fig. 7A. Nude 92-2. The tumor of HLC cells (10^7) shows lobular pattern of collagen fibers proliferation. Non-treatment with SSM. Control. 100 days. Pap silver stain, ×200.
Fig. 7B. The lobular proliferation of HLC cancer cells became degraded and disappeared due to confinement by remarkable proliferation of collagen fibers (45 days) after treatment with Z-100 (50 μg) at the same time as cell transplantation. Pap silver stain, ×200.

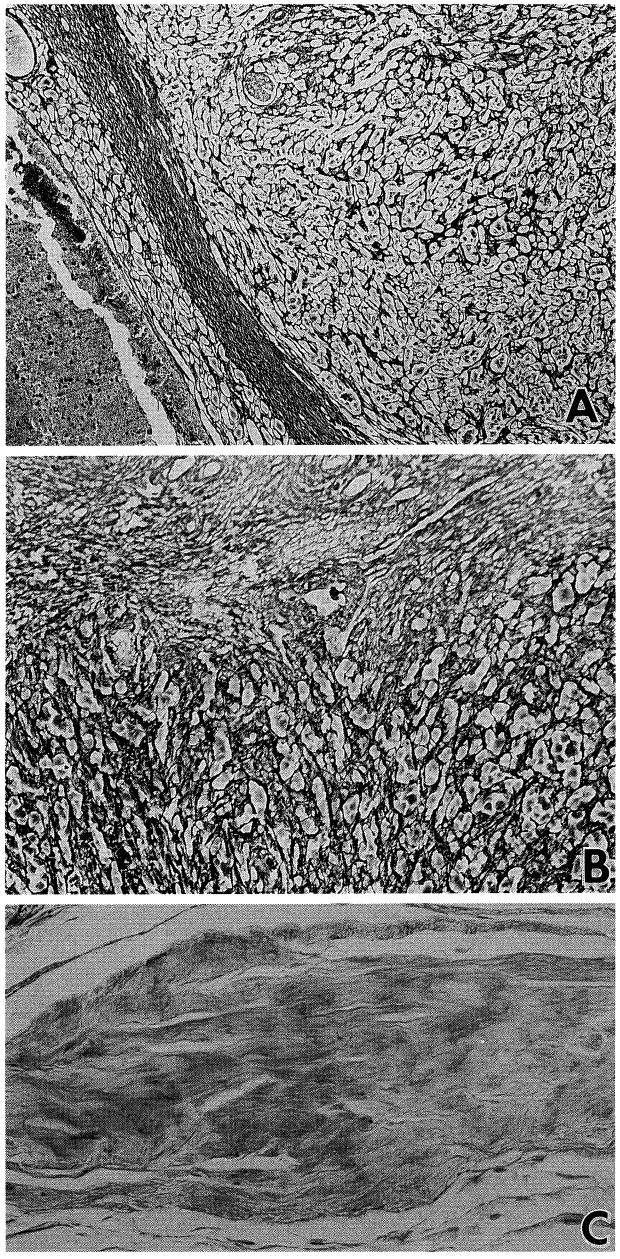


Fig. 8A. Nude 356-1. TRPC-9 cells (10^7) tumor was injected s.c. Z-100 (50 μg) for 26 days. Collagen proliferating patterrs show lobular and diffuse mixed types. Pap silver stain, $\times 200$.

Fig. 8B. Nude 357-4. TRPC-9 cells (10^7) tumor was injected s.c. Z-100 (50 μg) for 163 days. Tumor was cicatrized by remarkable proliferation of collagen fibers.

Fig. 8C. Nude 354-4. TRPC-9 cells (10^7) tumor was injected s.c. Z-100 (50 μg) for 133 days. The tumor was enveloped and completely replaced by collagen fibers. This is a mode of cure of cancer by collagen fibers. Mallory stain, $\times 100$.

with grafting of cancer cells and three times weekly thereafter.

Facilitation of collagenation and cicatrization of cancer lesion by long-term use of SSM and Z-100

As shown in Fig. 4, the proliferation of stromal collagen fibers developed

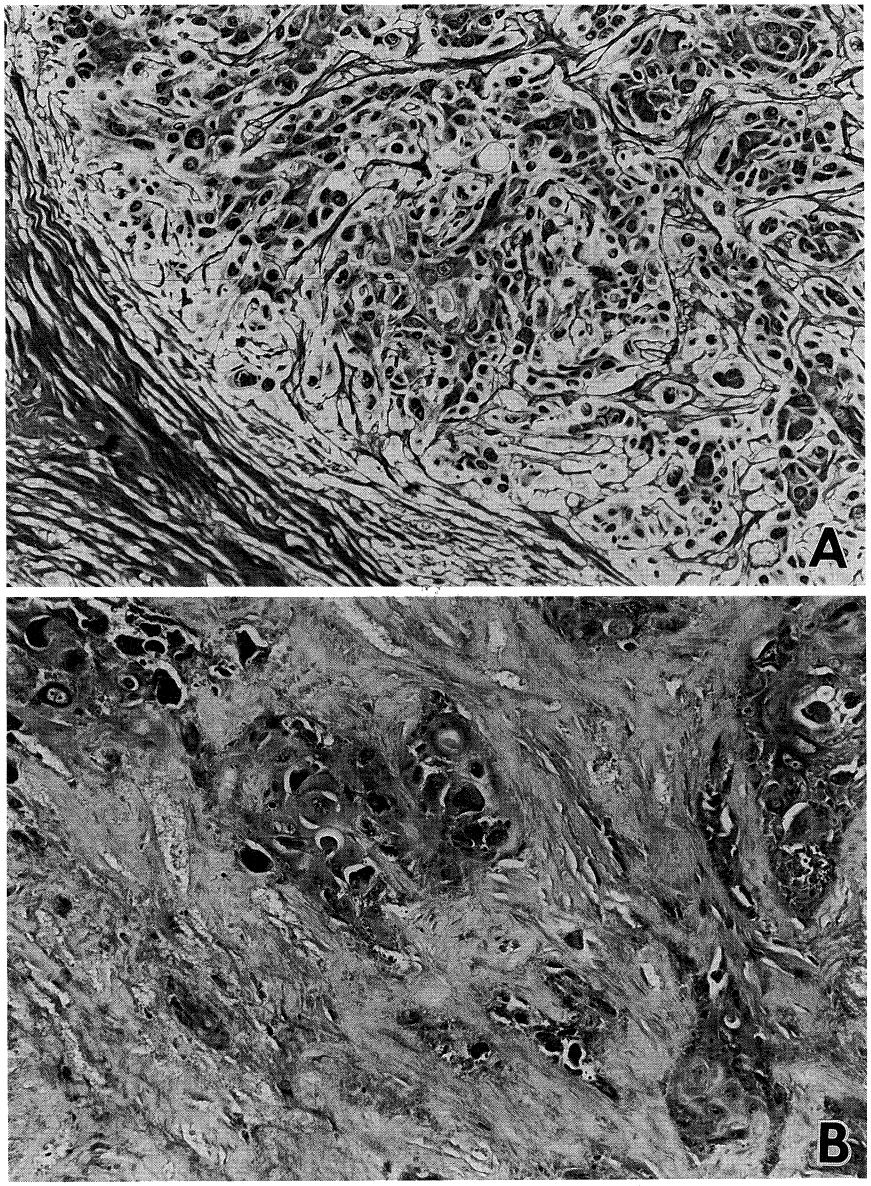


Fig. 9A. Nude 432-1. TRMKN cells (10^7) tumor, non-treatment with SSM and Z-100. Control. 112 days. Mixed type of diffuse and lobular patterns of collagenation.
Fig. 9B. Nude 446-1. TRMKN cells (10^7) tumor was injected i.t. SSM (1.0 μg) for 57 days. The tumor was cicatrized and healed for 57 days. Pap silver stain, $\times$200.

in grafted tumors was initiated by angiogenesis, and the cancer cells were surrounded by endothelia, fibroblasts, strokes collagen fibers to form a stromal architecture. The pattern of collagen proliferation thereafter, however, varied with cancer cell types,[11,19-22] particularly differences in cell membranes. Such collagen proliferation proceeded diffusely into intercellular space even in HGC undifferentiated cancers reported previously, and collagen fibers (positive to Mallory impregnation) developed around individual cancer cells 1 month later (Fig. 5A,B). When SSM was administered repeatedly for a long-term, collagen proliferation was facilitated, the cancer lesion finally became nodular, and cicatrized (Fig. 5B). In the case of HLC adenocarcinoma, on the other hand, either a tubular or an alveolar pattern developed (Fig. 7A,B), and diffuse proliferation towards the lobule, however, was not observed at an early stage. Long-term or high-dosed administration 100 μg of Z-100 facilitated collagen production from fibrous material leaking from the necrotic cancer lesion. This collagenation infiltrated into the lobule, leading to proliferation similar to that noted in liver cirrhosis, and the cancer cells were firmly encapsulated[6-8,11] (Fig. 7B). In the present study, stromal collagenation in well differentiated adenocarcinoma (epidermal carcinoma) and squamous cell carcinomas was particularly focused. Not only in primary squamous cell carcinoma but also in highly differentiated and metaplastic cancers such as TRPC-3 and TRPC-9 lung cancer and TRMKN-1 gastric cancer, stromal proliferation was predominant from an early stage of grafting, and collagen fibers were stained strongly in blue by Mallory's and Masson's methods (Fig. 8A). These collagen fibers gradually infiltrated in a tubular or alveolar pattern into the lobule to form a boxing pattern, and the diffuse and alveolar patterns finally coexisted (Fig. 8B). This marked collagenation was convarted to profound cicatrization by SSM

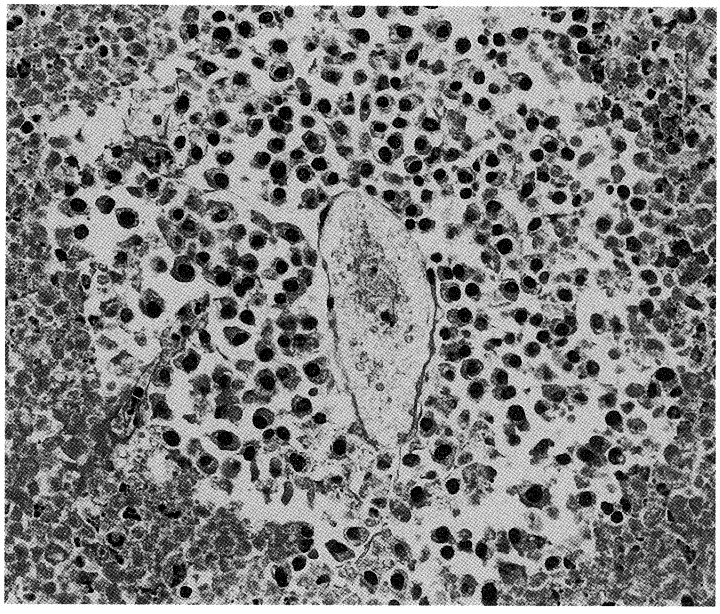


Fig. 10. Nude 285-1. TRPC-6 cells (2×10^7) tumor (oat cell cancer), non-treatment with SSM. Control. 71 days. Collagen proliferation was not seen or collagen fibers were very fine. H.E. stain, $\times200$.

and Z-100 injection, and then was shed (Figs. 2A,B,6). Thus an ideal cure of cancer due to cicatrization was achieved by collagen fibers (Fig. 8C). The collagen types in these xenografts were main components of types I and III (Fig. 9A,B). Collagenation from destroyed cartilages and calcification of necrotic cancer cells were observed as described in the previous report.[6-8]

Human small cell carcinoma (oat cell carcinoma) and murine Lewis lung carcinoma

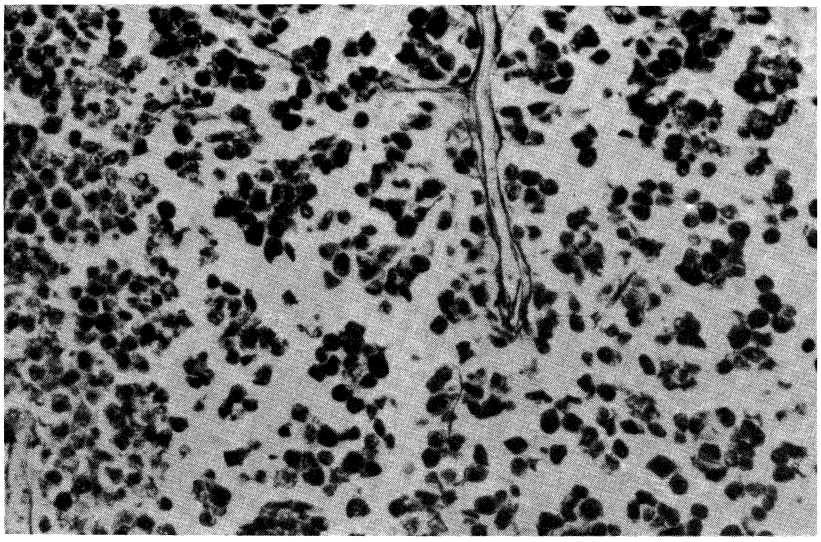
A cicatrized cure of cancer by collagenation was not observed in untreated control mice, but ulceration due to natural death of the cancer cells and resultant superficial cicatrization were noted in some control cases. In rapidly growing cancers such as undifferentiated human oat cell carcinoma and murine Lewis lung cancer, however, collagen fiber production was very few or absent (Fig. 10), and SSM was ineffective, allowing the cancer to grow rapidly to 5 cm in diameter. SSM failed to prolong survival of mice inoculated with small cell carcinoma or Lewis lung cancer, both of them were defficiency of intra-tumoral stromal reactions and collagen fiber proliferation, in nude and C57/black mice as shown the allografts of neuroblastoma (NB4A1).[8,11,20]

SSM (Z-100) has no direct cytocidal effect on cloning efficiency of cancer cells (Table 1)

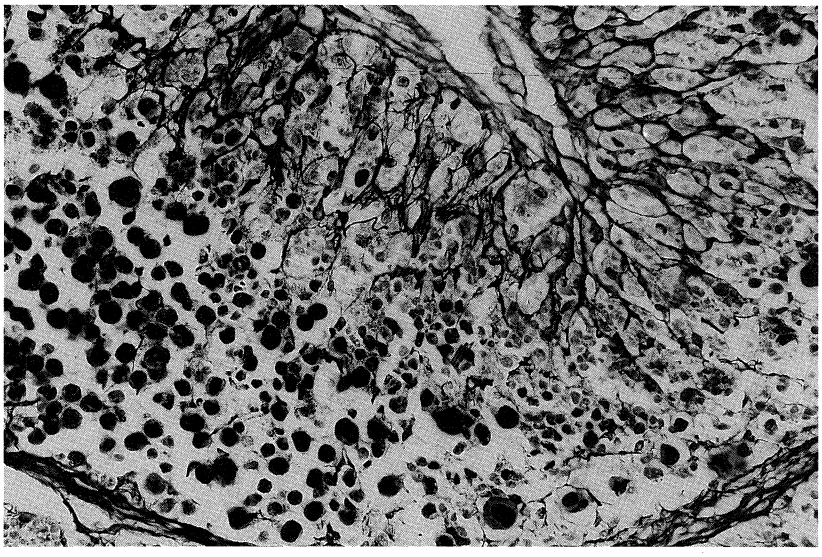
Two μg-200 μg/ml of SSM and Z-100 failed to inhibit the cloning efficiency of fibroblasts. SSM did not show any effect on the cloning efficiency of cancer cells at 2 μg/ml, but 30-50% inhibition was observed at 100 and 200 μg/ml (Z-100).

TABLE. 1. Cytocidal effects of various doses of SSM and Z-100 on human cancer cells and fibroblasts

Cells	SSM μg/ml Z-100	Plating Efficiency (%)	Percent of Control	Inhibition
HGC	0	64.5	100	0
	2	69.5	107.7	−7.7
	200	28.2	43.7	56.3
TRMKN-1	0	16.1	100	0
	2	25.9	161.3	−61.3
	100	10.4	64.5	35.5
HLC	0	17.8	100	0
	2	17.3	97.2	2.8
	200	9.8	54.9	45.1
TRPC-9	0	76.1	100	0
	2	76.1	100	0
	100	38.3	50.4	49.6
Human Skin	0	27.2	100	0
Fibroblast	2	25.0	92.5	7.5
	100	28.0	112	−12
Balb/c (whole embryo)	0	1.8	100	0
Fibroblast	2	1.6	88.9	1.1
	100	2.0	111.1	−11.1



A



B

Fig. 11A. Balb/c nu/nu. Allograft of NB41A3 cells (6×10^6). No response of collagenation from the blood vessels was observed and the tumor grew to the size of a hen's egg inspite of SSM treatment. Pap silver stain, ×200.

Fig. 11B. Balb/c nu/nu. Allograft of G.A. fixed NB41A3 cells (3×10^7) was transplanted subcutaneously. 17 days. Collagenation occurred from the walls of the blood vessels without the appearance of lymphocytes. Pap silver stain, ×400.

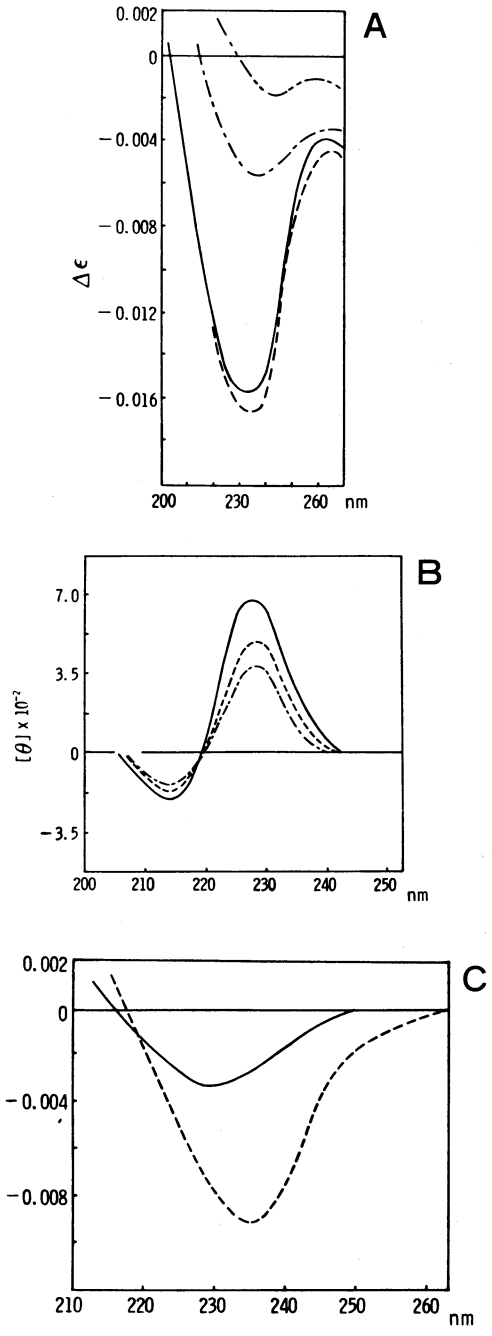


Fig. 12A. The circular dichroism spectra of native HGC (——), HLC (— — —), fixed HGC (— — — —) and HLC (— — —) with 0.25% glutaraldehyde.

Fig. 12B. The circular dichroism spectra of human fibronectin (0.72 μM) in the absence (——) or presence (– – –: 5.0 mM, — — —: 10.0 mM) of glutaraldehyde as a value of 114 for mean residue weight.

Fig. 12C. The circular dichroism spectra of native (——) and G.A. fixed human erythrocytes (– – –).

Cancer cells, which produce little or no collagen fibers, will be induced to proliferate collagen fibers by molecular conformational changes of glycoproteins on cell membranes

Transplantation of neuroblastoma cells of mouse origin in Balb/c nu/nu (athymic) mice[8]

Cultured neuroblastoma cells of mouse origin (NB41A3) had 100% transplantability when 3×10^7 cells were transplanted and they gave rise to distinct tumor. NB41A3 tumor grew easily, reaching 2 cm in 3 weeks. In this case there was no reaction in interstitial tissue to tumor cells, not to speak of the proliferation of collagen fibers from the perivascular area. Lymphocytes, mononuclear cells, and macrophages hardly appeared (Fig. 11A).

Transplantation of NB41A3 murine neuroblastoma cells fixed by 0.25% glutaraldehyde (GA)

No proliferation of collagen fibers derived from blood vessels took place at all against native NB41A3 cells in any host (Fig. 11A). When GA-fixed cells were transplanted, however, latticelike collagen fibers proliferated to encircle each cell near the blood vessel (Fig. 11B). This observation indicated that the collagen proliferation against transplanted cancer cells in the host depended not only on the type of these cells but also on the molecular conformation of their cell membrane. Circular dichroism (CD) was used to further clarify changes in the molecular structure.

Changes in molecular conformation induced by GA fixation as determined by CD

CD spectra at 200–260 nm of HGC and HLC cells treated with GA and of untreated native cells. The minimal absorption of essentially identical intensity was observed at 230 nm in both types of unfixed native cells, HGC and HLC. When fixed in GA, HGC and HLC cells showed one-ninth and one-third, respectively, of the minimal absorption of the unfixed cells. These differences seemed to have derived from the amount of cell surface component that was fixed with GA (Fig. 12A). The presence of fibronectin in HGC and HLC cells was confirmed by the indirect immunofluorescent antibody technique, and it was presumed that fibronectin was the component contributing to the decrease in molecular elliptical nature caused by the GA treatment. The CD spectra of highly purified fibronectin were measured at 200–250 nm in the presence and absence of GA. Fibronectin untreated with GA showed spectra in which the minimum and maximum were found at 214 and 228 nm, respectively, which were the same as those reported by Mosesson[23] (Fig. 12B). When the same material was treated with 5 μl of 10% GA for 5 min, its CD spectrum was reduced in intensity by about 28%. When the material was treated further with an additional 5 μl of 10% GA, its CD spectrum decreased further in intensity by 23%. The intensity of absorption at 228 nm was, thus, reduced in proportion to the amount of GA added, however, that at 214 nm was scarcely influenced by GA treatment. CD spectra of native erythrocytes, the surface of which had no detectable fibronectin, and of these cells after

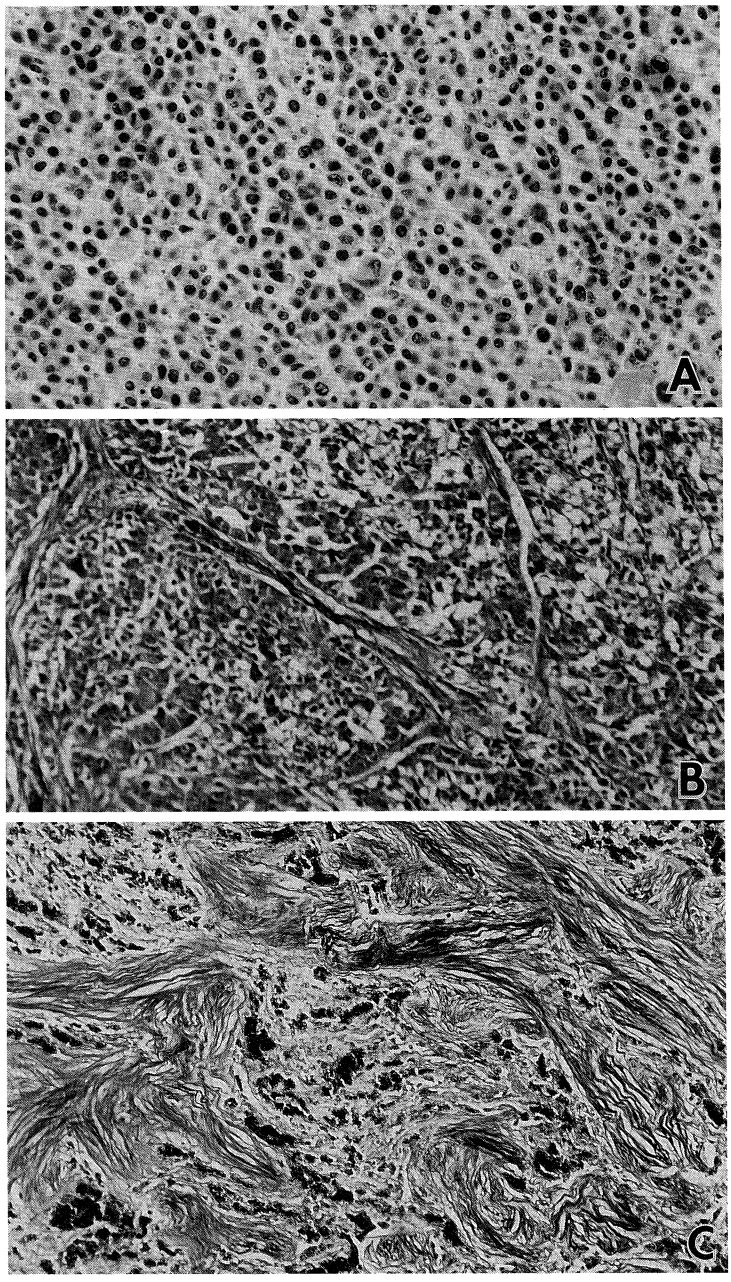


Fig. 13A. Nude 721-1. Rat hepatoma cells (10^5) tumor without the stromal formation was injected s.c. Z-100 (50 μg) for 33 days. Collagen fiber proliferation was not observed.
Fig. 13B. Nude 437-5. Rat hepatoma cells (10^5) tumor. Non-treatment with SSM. Control. 104 days. Stromal formation was observed in the tumor.
Fig. 13C. Rat hepatoma cells (10^5) tumor with stroma was injected i.t. Z-100 (100 μg) for 106 days. Although the tumor size was 28×20 mm, the tumor cells were confined and cicatrized by remarkable cirrhotic collagenation.

treatment with GA (Fig. 12C).

The fixed cancer cells were dead, had no ability to produce collagen fibers, and served only as a foreign body. Proliferating collagen fibers surrounding fixed cancer cells were in such a proximity that they seemed to have derived from these cells. We also found that in the case of HLC, native cancer cells were surrounded by lobular collagen fiber proliferation, whereas fixed HLC cells were surrounded by diffuse collagen fiber proliferation. This difference in the pattern of collagen formation may be related to the molecular conformational changes of the cancer cell membrane.

GA is regarded as the best of all fixatives now in use dye, primarily, to the fact that GA preserves the molecular conformation of the cell surface of native cells most effectively. When GA-fixed NB41A3 cells were transplanted, collagen fibers proliferated without any lymphocyte and macrophage infiltration. When living native NB41A3 cells were transplanted there was no stromal reaction. Since fibronectin was present on the surface of HGC, HLC, and NB41A3 cells, we suggest that the changes in CD of HGC, HLC, and NB41A3 cells after GA treatment was related to fibronectin,[24] and that fibronectin may be an important key in clarifying the relationship between cancer cells and collagen proliferation.

Effects of SSM and Z-100 on rat hepatocellular carcinoma (hepatoma) with or without stromal formation in nude mice

This is clearly shown by the character of cancer cells which is made from rat's liver with ferric nitrilotriacetate (Fe-NTA) by Yamada, Okigaki and Awai.[25] Pathological findings of this hepatoma cells were grafted into nude mice showed hepatocellular cancer with and without interstitial tissue (Fig. 13A,B). As shown in Fig. 13B,C collagenation by SSM and Z-100 injection was remarked in the hepatoma with stromal formation and hepatoma cells were encapsulated or confined by collagen fiber proliferation (Fig. 13C), on the other hand, collagen fiber proliferation in the hepatoma without stroma was not observed in cancer lesions, in spite of the treatment with Z-100 (Fig. 13A).

Conclusion concerned various cancer cell xenografts

As shown in Fig. 4, SSM activates macrophages, NK-4 cell, T-cell and produces a quantity of cytokines (such as IFNs) in vitro.[15-18] Increased collagenation and fibroblast proliferation are observed even in athymic nude mice.[12,13] Therefore, we believe that SSM activates macrophages directly or indirectly by stimulating NK-cells, dentric leucocytes system, or T-cells.

In a cancer lesion, a microenvironment of cancer cells and interstitial cells are reconstructed, and BRM is not implicated in this formation. The degree of collagen fiber production is different in each cancer cell and depends on the molecular conformation of the glycoproteins such as fibronectin on the cancer cell membrane or excellular matrixes. Cancer cells, which shows more remarkable collagen proliferation in a cancer lesion without SSM, spontaneously responds to SSM more remarkably. SSM does not affect cancer without stromal reaction such as oat cell carcinoma, Lewis lung cells and neuroblastoma cells (Figs. 10,11A,13A). Chemotherapeutics can cause cancer necrosis

and SSM cause cicatrization of the cancer by promoting the healing of necrotic lesion.

We conclude that the antitumor activity of SSM is not based on an ability to kill cancer cells, but rather on tissue repairing ability to promote collagen proliferation in the cancer lesion. This causes encapsulation of the cancer lesion, thereby inhibiting cancer development. As a result, the cancer may degrade or sometimes even disappear entirely. This enables the survival expectomy to be lengthened even when cancer is present. This mechanism is pathologically similar to the mechanism for healings of wounds or tuberculosis.

Regression of growth of malignant melanomas grafted onto athymic and thymic mice[18]

In 1968, Mathe[26,27] reported a case of acute lymphocytic leukemia treated with Mycobacterium bovis BCG, malignant tumor treatment with BCG began to draw interest. Then, Bluming *et al.*[28] described the remarkable effect of BCG on malignant melanomas. Subsequently, in Japan, Yamamura *et al.*[29] reported on investigations of malignant melanomas and of carcinomatous pleurisy treated with BCG-CWS (the cell wall basic substance of Mycobacterium bovis).

Recently, however, in studying the anti-cancer effect of SSM on tumors of human-derived cultured malignant melanoma cells and mouse-derived malignant melanoma cells grafted onto athymic and thymic mice, we found that it also inhibits the growth of malignant melanomas.[21]

Cell lines

TRG361, a human-derived malignant melanoma cell line obtained by first grafting 3×10^7 G361 cells subcutaneously and then reculturing implanted tumors, and clone-M-3 and B-16 cell lines, two mouse-derived cell lines, were used as experimental materials. All these lines were purchased from Dainippon Pharmaceutical Co., Ltd., Japan.

Grafting

TRG361 cells were grafted onto athymic mice (Balb/c nu-nu) and clone-M-3 and B-16 cells also onto athymic mice (Balb/c nu-nu) and thymic mice (Balb/c and C57 black/6). Clone-M-3 and B-16 took 100% on the mice and grew in $2.5\text{-}6\times10^6$ cells, and TRG361 took and grew in 10^7 cells.

SSM and Z-100 injections

Simultaneously or three days after the cell grafts, 0.5 μg, 1.0 μg, 50.0 μg, or 100.0 μg of SSM and Z-100 were injected s.c. or i.t. every other day.

Antitumor effect on malignant melanoma growth in thymic and athymic mice

Clone-M-3 graft experimental group

Thymic mice grafts

In mice receiving SSM 1.0 μg s.c., cicatrization accelerated 50 days after

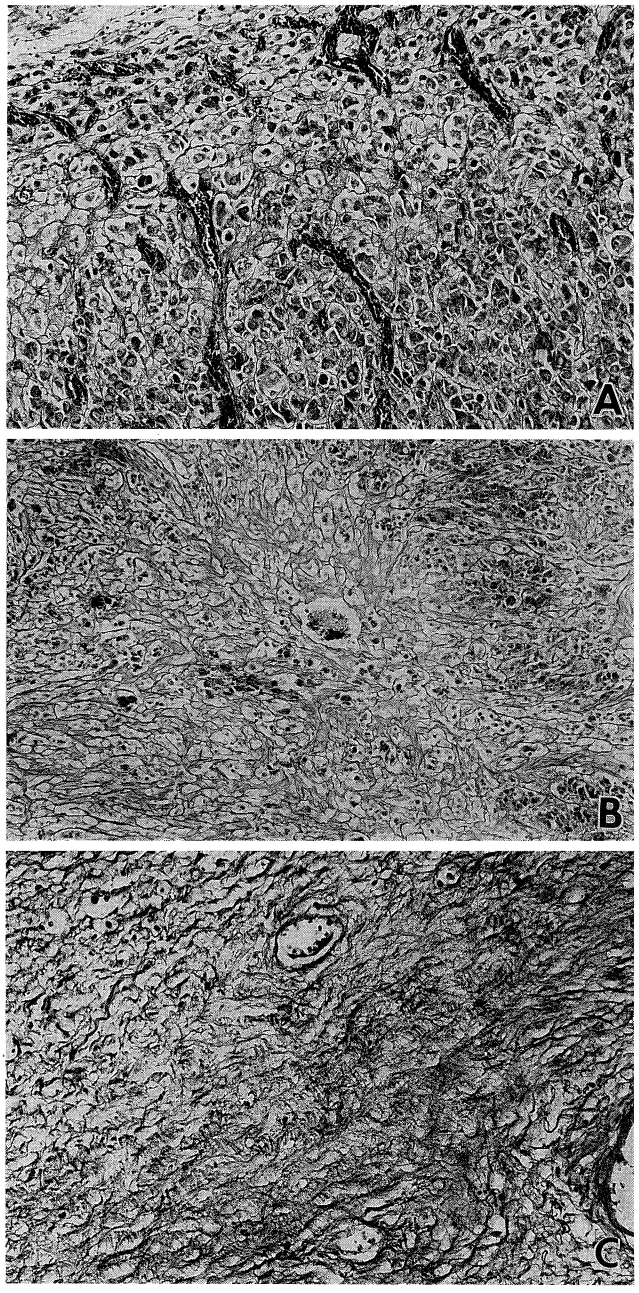


Fig. 14A. Nude 428-1. Clone M-3 cells (10^7) tumors of the nude mouse. Angiogenesis in the tumor was remarkable. 10 days.

Fig. 14B. Balb/c thymic mouse 408-2. Clone-M-3 cells (10^7) tumor was injected s.c. SSM (2 μg) for 67 days. Diffuse collagenation was remarked in the tumor and the tumor was cicatrizing.

Fig. 14C. Balb/c thymic mouse. 425-5. Clone-M-3 cells (10^7) tumor was treated with Z-100 (50 μg) s.c. for 162 days. Tumor was replaced by collagen, cicatrized and healed.

grafting (10^7 cells), and tumor cicatrization was observed 150 or more days after grafting in the group injected with SSM at the time of grafting. Tumor cicatrization or abrasion was especially observed after injection of SSM 50 μg i.t.

In contrast, in the thymic mouse, control group receiving no injection, tumors expanded to 40-50$\times$50 mm 100 days after 10^7 graft without cicatrization.

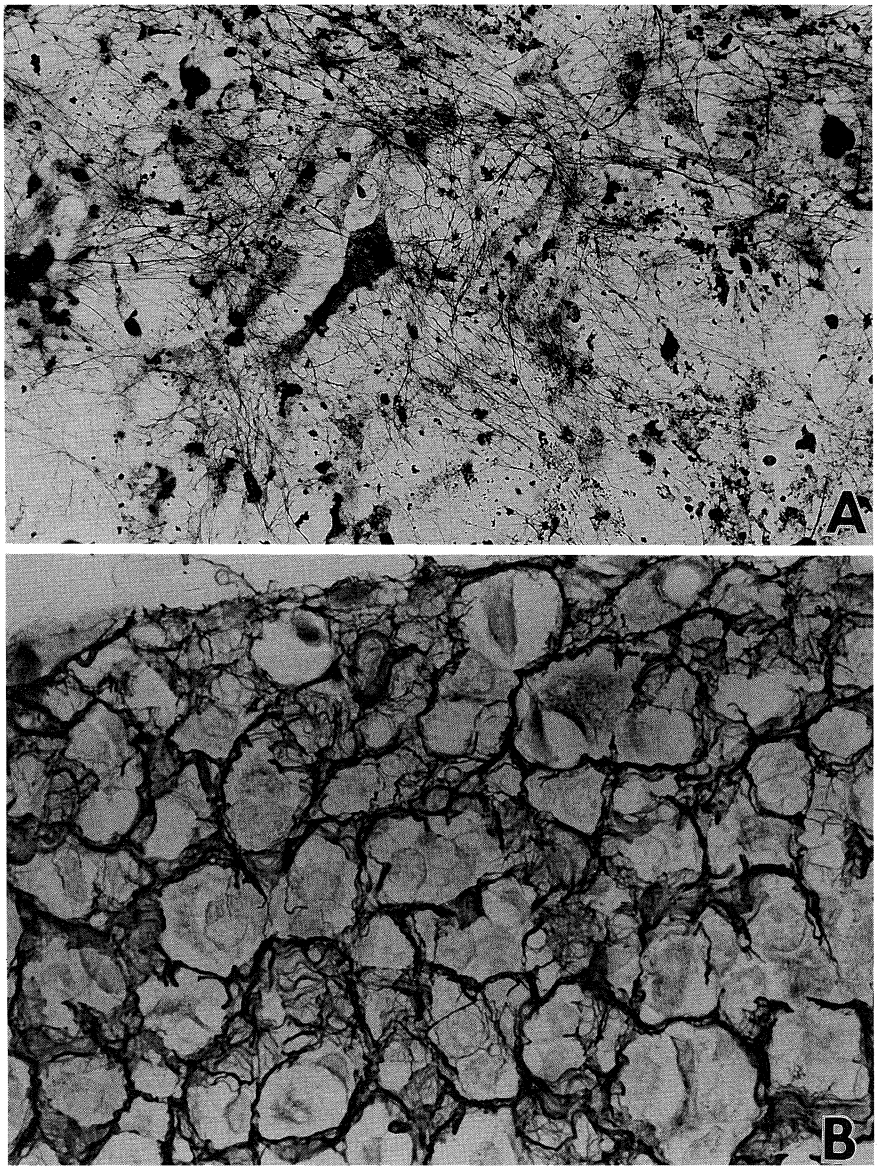


Fig. 15A. In vitro collagen fiber production and proliferation in co-cultivation with C3H mouse fibroblasts and clone-M-3 cells. 4 weeks.
Fig. 15B. Nude 336-5. In vivo (nude mouse) collagen fiber proliferation (IV type) in clone -M-3 (2×10^7) tumor. 7 days. Pap silver stain, $\times$400.

Athymic mice grafts

Tumors expanded to 35×30 mm 40 days after the 1×10^7 graft, and no trend of natural cure was observed in the untreated control group.

On the other hand, in a group injected i.t. with Z-100 50.0-100 μg at the time of the 1×10^7 graft or another group injected s.c. with the same amount of Z-100, regression of tumor growth and cicatrization were observed. However, the degree of cicatrization and the speed with which it occurred was more pronounced in mice injected i.t.

Histological findings of clone-M-3 allogeneic grafts

Two types of melanoma cells were recognized histologically. One was a spindle type (fibroblastic type) and the other was an epithelial or tubular type.

In the malignant melanoma cell graft, angiogenesis was especially conspicuous (Fig. 14A). Tumors increased with newly formed blood vessels in the center of the tumor. The tumors showed foliate growth and a marked circulatory disturbance occurred in the area around the lobule due to vessel reconstruction accompanying tumor cell growth. This lesion underwent natural death.

Stromal formation was completed about 1 week after grafting (Fig. 15B). Compared with previously reported cases of cancer cell grafting,[21] the growth of Mallory stained positive collagen was weak, but fine collagen fibers originating with angiogenesis increased diffusely 5 days after grafting. Collagen type IV[30] extended in a mesh-like pattern into the tumor (Fig. 14B). Collagen type III and then type I were found distributed in the original stroma. Collagenation became conspicuous as the SSM treatment period was prolonged to 50 or more days after grafting.

Three days after grafting, macrophages and polynuclear leukocytes[31,32] appeared transiently in the stroma.

SSM (Z-100) treatment of 50 μg promoted greater collagenation. Although injection of SSM (Z-100) i.t. was particularly effective, subcutaneous injection was also effective in long-term use. Since SSM (Z-100) exhibited a cytotoxic effect when used in high concentrations in vitro, the possibility of a cytotoxic effect from intratumor injection was considered. However, marked growth of reticular fibers (collagen IV type) due to the SSM (Z-100) injection began about 40 days after grafting and cicatrization was clearly promoted. Collagenation in thymic mice was remarkably promoted by injection of SSM (or Z-100) (Fig. 14B,C). Even SSM 0.5 μg injected s.c. resulted in greater collagenation and a longer life than in athymic mice. Tumors cause cicatrization due to cell death and collagenation. In the untreated group, collagen proliferation was extremely weak even 90 days after grafting.

B-16 graft experimental group[21]

Thymic mice (C57BL/6)

In B-16 (2×10^7) grafts, tumors expanded remarkably and grew to 40×30 mm 40 days after grafting. The untreated control group showed no indications of recovery nor did the group injected with SSM (Z-100) 1.0 μg-50 μg s.c.

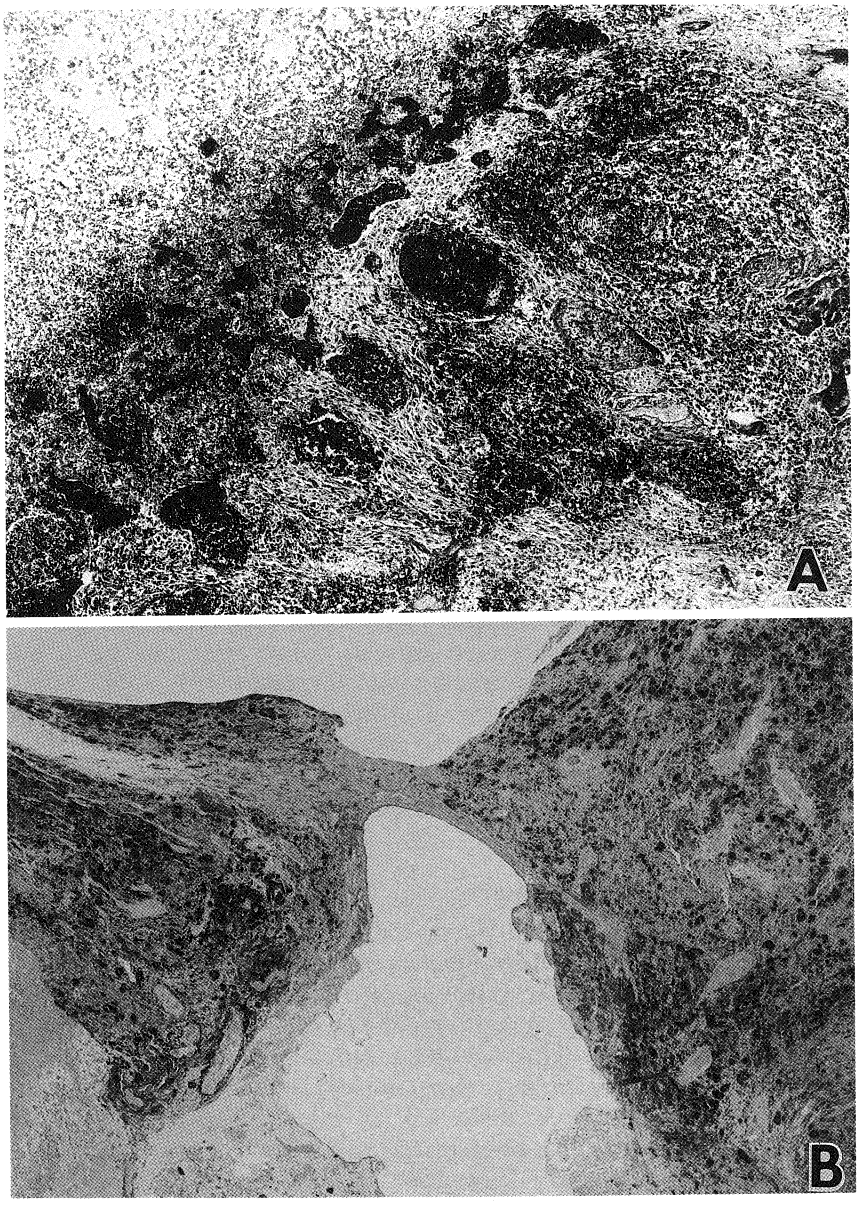


Fig. 16A. Nude 456-2. B-16 cells (5×16^6) tumor was injected i.t. SSM (1.0 μg) for 21 days. Remarkable angiogenesis and hemorrhage were observed in the tumor. Mallory stain, × 200.

Fig. 16B. Nude 450-4. B-16 cells (5×10^6) tumor was injected i.t. Z-100 (50 μg) for 66 days. Tumor was cicatrized. Cystic necrosis by cytocidal effect of SSM was observed in the tumor. H.E. stain, ×200.

When injection of SSM 50 μg i.t. was started at the time of grafting, growth regression was observed and the tumor size was about 20×15 mm 30 days after grafting in most cases.

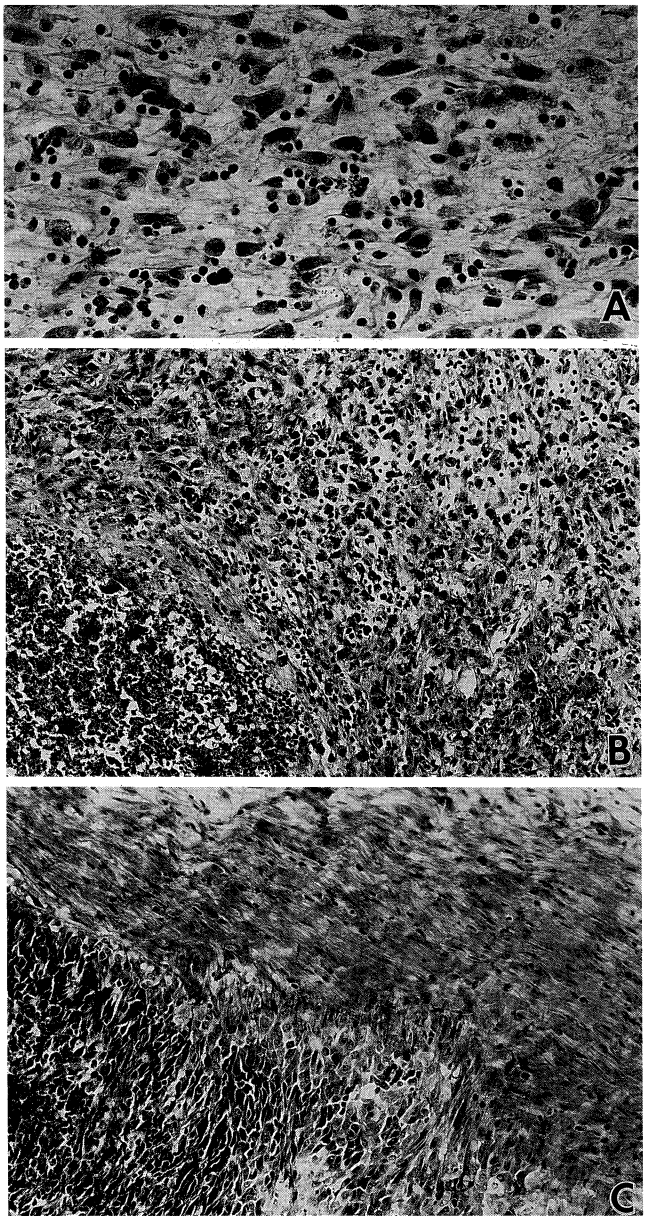


Fig. 17A. Nude 405-2. B-16 cells (5×10^6) tumor was injected s.c. Z-100 (100 μg) for 39 days. Remarkable macrophages proliferation can be seen in progress. In the stroma, collagen proliferation and lymphocytes infiltration also can be seen in the nude mouse. Mallory impregnation, $\times400$.

Fig. 17B. Nude 487-3. B-16 cells (5×10^6) tumor was injected i.t. Z-100 (100 μg) for 54 days. In the stroma collagen fiber proliferation accompanied by macrophages and lymphocytes proliferation was remarked. Mallory impregnation, $\times200$.

Fig. 17C. C57BL/6 617-1. B-16 cells (5×10^6) tumor was injected i.t. SSM (1.0 μg) for 21 days. Remarkable proliferation of fibroblasts and collagen fibers accompanied by macrophages proliferation can be seen in the stroma. Mallory impregnation, $\times200$.

Athymic mice (Balb/c nu-nu)

No marked effect was observed with a B-16 2×10^6 graft and simultaneous administration of SSM 1.0 μg s.c., but cicatrization accelerated 60 or more days after grafting with early injection of SSM. In mice injected with SSM 50 μg i.t., rapid cicatrization was observed at a high rate 60 days after grafting.

In the untreated control group, tumors expanded and infiltrated deep tissues and reached a size of 40×40 mm 50 days after grafting in $2\text{-}5\times10^6$ grafts.

Histological findings of B-16 allogeneic grafts

Angiogenesis was the most remarkable findings in both athymic and thymic mice (C57BL/6), and is indicative of tumors with a strong tendency toward metastasis (Fig. 16A). Although collagenation was strongest in newly formed blood vessels and collagen proliferation from the stroma was still weak up to 30 days after grafting, a double stromal layer had formed 20 days after grafting, and marked proliferation of fibroblasts occurred following injection of SSM and Z-100. As in the clone-M-3 graft, growth and proliferation of Pap argentation positive reticular fibers (collagen type IV) was seen in tumors (Fig. 15B). Noticeable migration and accumulation of polynuclear leukocytes as Brocker *et al.*[31] and Ishikawa *et al.*[32] described were seen about 30 days after grafting in the double layer structure of the stroma, especially in the inner layer (neovascular area) of the grafted tumor and the interstitial boundary.

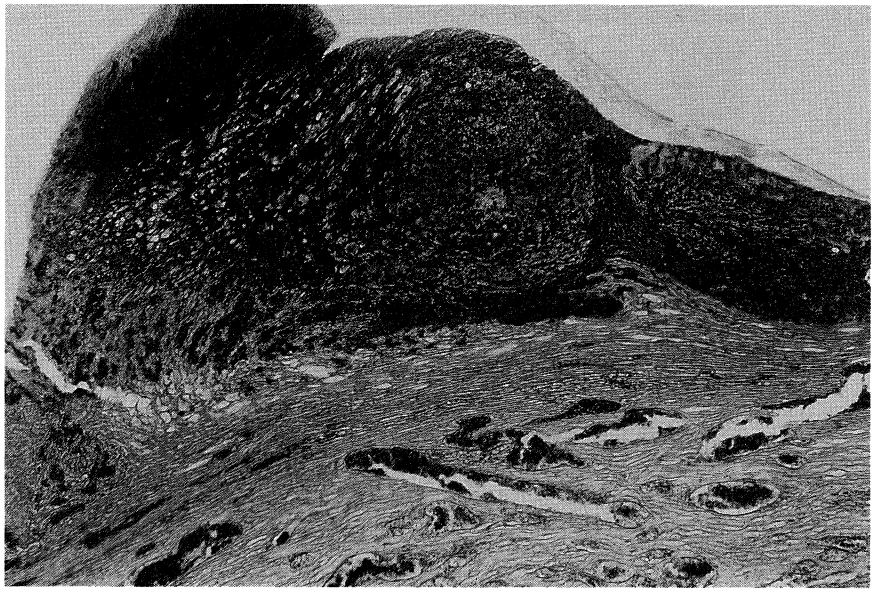


Fig. 18. Nude 462-3. B-16 cells (5×10^6) was transplanted, and concurrently, SSM 100 μg was injected s.c. every other day for 55 days. Tumor cells, which caused vacuolation and necrosis, were observed between remarkable tumor cicatrization and collagen proliferation. Remarkable collagen proliferation and necrotic tumor cells were also observed in the interstitium. Pap silver impregnation, $\times400$.

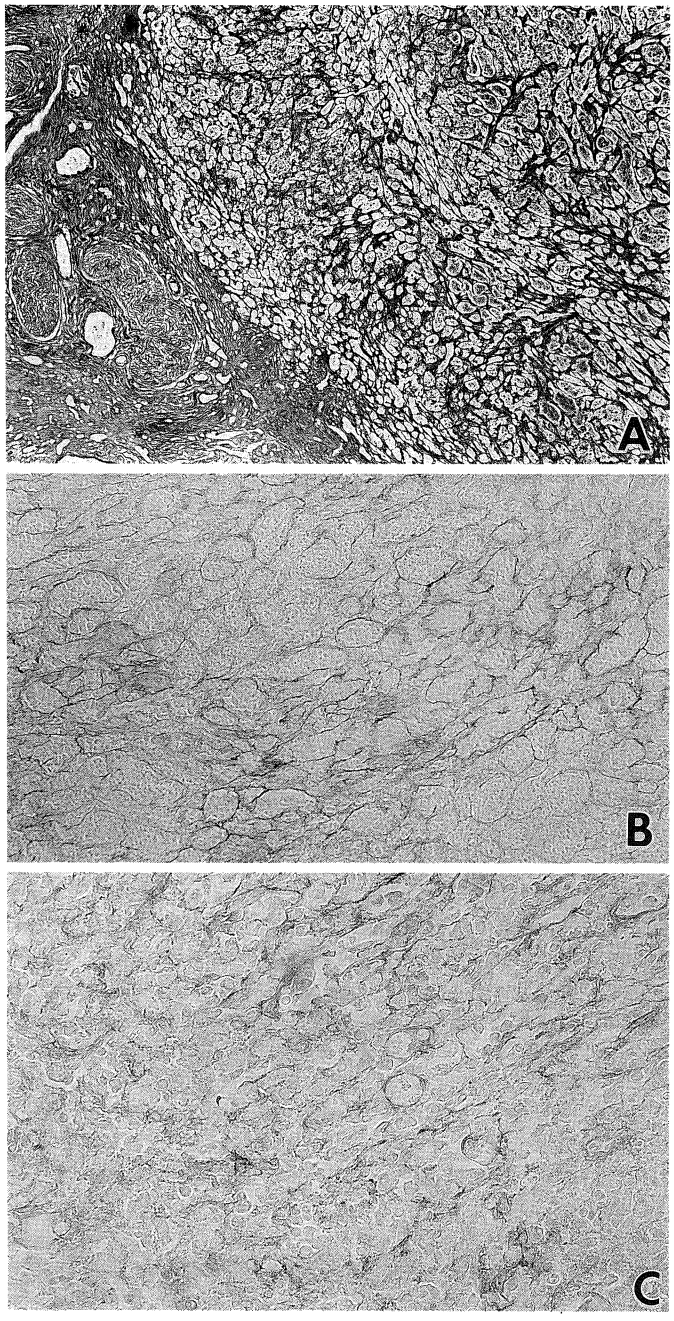


Fig. 19A. Nude 329-4. TRG3631 cells (10^7) tumor was injected s.c. Z-100 (50 μg) for 102 days. Collagen fiber proliferation showed diffuse pattern into the tumor. Pap silver stain, ×200.

Fig. 19B. Nude 329-4. Immuno-peroxidase stain with antibody treatment showed IV type collagen in the tumor. ×200.

Fig. 19C. Nude 329-4. Fibronectin also was observed in the tumor by Immuno-peroxidase stain with anibody treatment. ×200.

With injection of SSM (Z-100) 0.5-100 μg i.t., macrophage proliferation also became noticeable 20-40 days after grafting (Fig. 17A,B). Simultaneously, in the stroma, collagenation became noticeable in the boundary of the tumor lesion, and its invasion of and proliferation into the tumor were observed (Fig. 17C).

While B-16 tumor cells grew extremely fast, intratumor injection of high concentrations of SSM (Z-100) caused an extensive cytotoxic effect (Fig. 16B). With the development of necrosis, collagenation was promoted due to these stromal reactions. Regression of tumor growth was observed about 50 days after injection, and cicatrization followed ulcer formation (Fig. 18). In this case, as well, collagenation was more striking in thymic mice than in athymic mice[18] (Fig. 17B,C).

TRG361 graft experimental group

Tumors reached 30×30 mm about 70 days after a 1×10^7 grafts in the control group and showed no trend toward natural recovery.

In the group injected with SSM (Z-100) 50-100 μg s.c. or i.t. at the time of grafting, tumor growth stopped 50 or more days after grafting, and cicatrization gradually proceeded 80 or more days after grafting. In the group injected with SSM 0.5-1.0 μg s.c. or i.t., the progress of cicatrization was slower, but the mice lived 2-3 times longer than those in the control group.

Histological findings of TRG361 xenografts

Human-derived 1×10^7 cells took in all athymic mice and reached the size of an egg 3 months after grafting.

Angiogenesis of almost the same degree as that in clone-M-3 was observed and tumor growth was relatively fast. Although ribbon-shaped growth of collagen was first seen around blood vessels, collagen proliferation around the vessels was promoted mainly by SSM injection (Fig. 19A). Like clone-M-3 cells, TRG361 cells exhibited two cell types-epithelial and fibroblast. Growth of intratumor collagen type IV was seen in tumors (Fig. 19B), after which growth of stromal collagen types I and III was noted. Fibronection was recognized in tumors (Fig. 19C), although its presence was limited.

When high concentrations (50-100 μg) of SSM (Z-100) were injected into tumors, proliferation of mast cells and macrophages were observed in the stroma, and migration of neutrophils was seen. Collagen proliferation also became marked encapsulation and cicatrization of tumor lesion were observed. Stromal collagen types I and III, intratumor collagen type IV, and intratumor fibronectins increased.

Cytocidal effect on malignant melanoma (Table 2)

Although SSM (Z-100) had only a weak effect on cancer cells, it had a strong cytocidal effect on malignant melanoma cells, as shown in Table 2. In particular, 2.0 μg of SSM had little cytocidal effect on melanoma, but 200 μg (Z-100) killed nearly 70% of TRG361 and B-16 cells, and nearly 100% of clone-M-3.

TABLE. 2. In vitro cytocidal effects on malignant melanoma cells by SSM and Z-100

Cells	SSM μ/ml	Plating Efficiency	Percent of Control	Inhibition
TRG-361	0	48.2	--	
	2	44.3	92.0	8.0
	200	14.3	29.7	70.3
Clone-M-3	0	10.0	--	
	2	6.7	66.7	33.3
	200	0.1	1.3	98.7
B-16	0	55.8	--	
	2	60.3	108.1	-8.1
	200	16.8	30.0	70.0

Histochemistry of collagen and fibronectin

As has been indicated earlier with regard to the kinetics of collagen and fibronectin in malignant melanoma tumors, collagen type IV growth was observed in tumors even in the untreated control group of athymic mice 5-7 days after grafting. In the original stroma, collagen types I and III were also observed, and fibronectin was seen locally in tumors. In all the groups treated with SSM (Z-100), the growth of collagen type III was conspicuous in the stroma about 60 days after grafting, after which growth of type IV, fibronectin and type I, respectively, was seen.

Conclusion concerned malignant melanoma

In this investigation we studied the antitumor effects of SSM (Z-100) on grafted malignant melanoma cells. A human-derived cell line, TRG-361, was grafted onto athymic mice, and two mouse-derived cell lines, clone-M-3 and B-16, were grafted onto athymic and thymic mice, respectively. SSM (Z-100) was injected every other day. Although all the cell lines exhibited a notable tumor angiogenesis, it was especially conspicuous in B-16.

After grafting, collagen type IV was found mainly in the tumors and collagen types I and III in the original stroma.

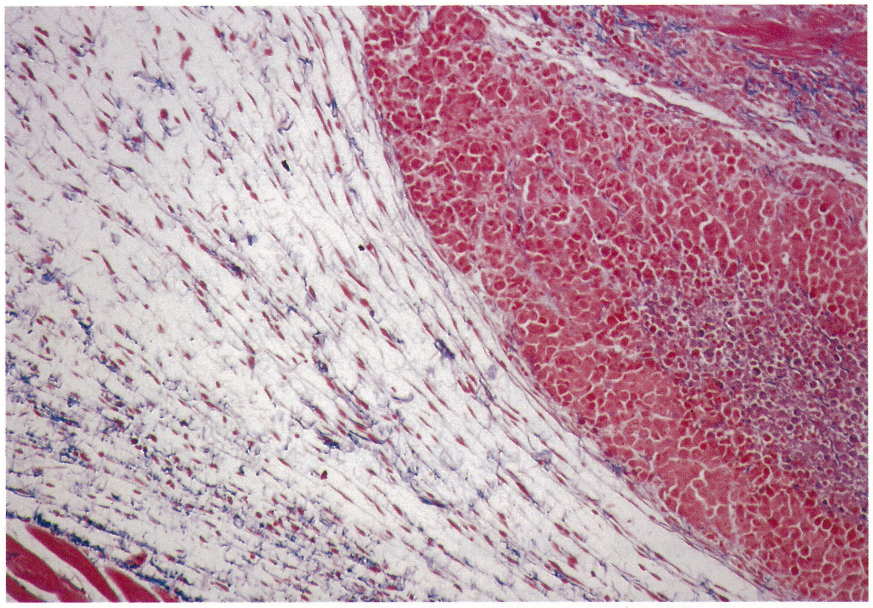
Injection of high doses of SSM (Z-100) promoted extensive cytocidal effect and collagenation. The tumor confinement induced by collagen proliferation suggests the importance of an antitumor effects by SSM (Z-100) on regression of growth of malignant melanomas.

The anti-tumor effects of SSM (Z-100) on MC-1 and IMC tumor cells allografts

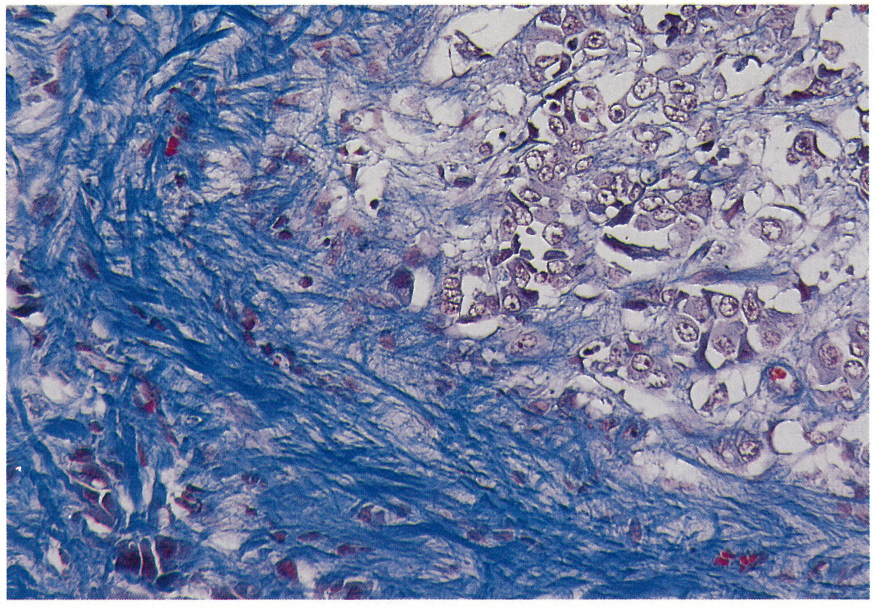
C57BL/6 mice were grafted with MC-1 sarcoma cells, which had been induced by 3-methylcholanthrene or IMC sarcoma cells obtained from spontaneous breast cancer of CDF1 mice. SSM antitumor effects, affected by BCG sensitization, were studied.

The anti-tumor effects of BCG are believed to be nonspecific reactions through the activation of macrophages. C57BL/6 male mice, 8-10 weeks of age, were sensitized by BCG.

0.1 mg or 1.0 mg of attenuated BCG per mouse was administered abdominally to make BCG-sensitized (after three weeks) mice for this



A



B

Fig. 20A. IMC cells (5×10^6) tumor. Saline i.t. injection. Control. 14 days. Mallory stain, $\times200$.

Fig. 20B. IMC cells (5×10^6) tumor. Z-100 (20 μg) i.t. injection. 14 days. Predominant proliferation of collagen fibers was observed in the stroma and tumor regression was induced. Mallory stain, $\times200$.

experiment.

Mice were grafted 10^5 MC-1 cells into the right inguinal region and divided into the following groups.

Control : 1) BCG+saline
2) untreated

SSM (Z-100) injection: BCG+SSM (Z-100), which was injected subcutaneously or into a tumor at a dose level of 10 mg/kg body weight. Seven days, 14 days, and 2 months after dosing, the major axis and minor axis were measured by dial calipers to determine tumor size (mm^3) and assess anti-tumor effects. Mice were grafter 10^5 IMC sarcoma cells into the right inguinal region

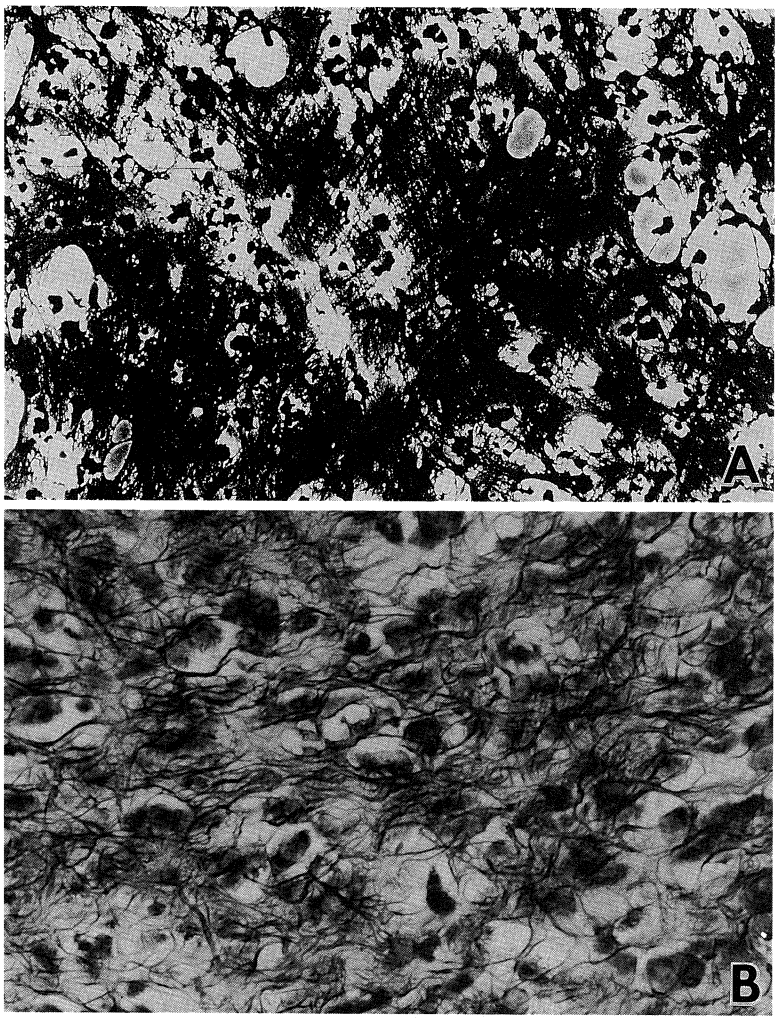


Fig. 21A. In vitro collagen fiber production and proliferation. Co-cultivation with Detroid 550 fibroblasts and HGC cells. 3 weeks.

Fig. 21B. HGC cells (2×10^7) tumor of Balb/c nu/nu mouse. SSM (2 μg) s.c. treatment. 70 days. Collagen fibers proliferated surrounding cancer cells. This finding was similar with in vitro collagen fiber proliferation surrounding cancer cells.

for the experiment.

Control : only saline injection

SSM (Z-100) injection : SSM (Z-100), which was injected subcutaneously or into a tumor at a dose level of 10 mg/kg body weight. Seven, 14 and 25 days after posing, the mice were killed and the sections of their tissues were observed.

MC-1 is one of the tumors which has responded consistently to BCG. In BCG-sensitized mice without cancer, the anti-tumor activity of macrophage was observed, while in BCG-sensitized mice with cancer, the anti-tumor activity of macrophage decreased and the production of killer T-cells, which react specifically to a tumor antigen, was promoted. Therefore, in a cancer bearing animals, a certain mechanism implicated in the production of killer T-cells, which react specifically to a tumor antigen, might exist. When BCG-sensitized mice with cancer are injected with SSM, the anti-tumor activity of macrophage will increase again. It is suspected that in BCG-sensitized mice which are injected with SSM (Z-100), the functions of both killer T-cells and activated macrophages will be stimulated.[15,18]

The stimulated functions of these cells by a high concentration of SSM (Z-100) caused remarkable inhibitory and degenerative effects on the grafted tumors within a short period of time (25 days).

Histopathological changes in these mice were also investigated. As shown in the Fig. 20A,B, although lymphocytes and macrophages were rarely observed, remarkable collagenation was produced in the interstitium around the tumor by activated nonspecific latent effecter cells (NK-cell, macrophage). Inhibitory effects on tumor proliferation were implicated in the degree of collagen proliferation, rather than cytotoxicity. The degenerative ratio was related to SSM concentrations, suggesting pathologically that interstitial collagen proliferation in inhibiting cancer development plays a more important role than cytotoxicity by using SSM (Z-100). Increased collagen proliferation in mice pretreated with BCG suggests a relationship between cancer and tuberculosis in humans. It is suggested that SSM (Z-100) might be more effective in human cancer than in mice without tuberculosis.

Disucssion and Conclusion

Collagen is produced and proliferates through cell-to-cell contact in a mixed culture of fibroblasts and either cancer cells or malignant melanoma cells in vitro (Figs. 15A,21A). Besides our report,[33] reported collagen fiber production and cytotoxicity against cancer cells in HeLa cells, and recently, Imanishi *et al.*[34] reported tumor-degenerating factors (TDF) such as glycoprotein, which is secreted as non-immune cytokine by fibroblast.

In addition, Sakakibara *et al.*[35] proved that even liver cells, which are epithelial, can synthesize and product collagen fibers. Sakakibara *et al.*[36] showed gastric cancer cells (scirrous) and breast cancer cells also produce collagen fibers. Langness *et al.*[37-40] also reported collagen synthesis in non-fibroblastic cell lines. We examined the ^{3}H-hydroxyproline intake of PC-3 (lung cancer), PC-9 (lung cancer), HLC (lung cancer), and HGC (gastric cancer) to study collagen biosynthesis; we clarified that these cancer cells

synthesize collagen, but do not release it from the cells. Therefore, considering the before mentioned behavior of cancer in a host, it is believed that the neovascular system through angiogenesis in cancer lesions are restructured, then the cancer lesion is surrounded with endothelial cells, which form a micro-environment enabling cancer and interstitial cells, especially endothelium and fibroblast, to contact each other (Figs. 15,21). Significantly, Sato and Suzuki *et al.*[41] have reported that a cancer blood flow lacks selfcontrol of blood flow seen in normal tissue. The degree of collagen production and proliferation depends on the characteristics of cell membranes of the cancer cells. However, immune cells were not essential for this collagen production and proliferation when stromal and cancer cells coexist. In cancer bearing host, rapidly proliferating cancer cells release protease such as collagenase, which breaks down the collagen fibers and causes the increase and infiltration of cancer cells, resulting in metastasis. Stromal tissue in cancer lesion plays an important role in increasing collagen synthesis and proliferation. This is clearly shown by the characteristics of liver cancer cells which is induced from rat's liver with ferric nitrilotriacetate (Fe-NTA).[25] As shown in Fig. 13C, collagenation by SSM (Z-100) injection was more remarkable in the hepatoma with the stroma and it is quite alike fibromellar carcinoma. It was suggested that (Z-100) was the treatment of choice and effective for cancer combined with stromal formation.

Based on the pathological findings of cancer mentioned above, BRM also plays an important role in stimulating collagen proliferation. In an experiment on athymic mice with cancer cell graft, in spite of lack for matured T-cell,[17,18] collagen proliferation was increased by SSM, especially Z-100. Besides macrophages, the dentric leucocytes, reticuloendothelial system (RES) and activated NK-cell might contribute to this phenomena.

Experiments which compared the effects of SSM (Z-100) on collagenation in malignant melanoma between athymic mice and thymic mice yielded the following results: The effects of SSM on collagenation was remarkably increased in thymic mice, where the migration of many lymphocytes, macrophages, and mast cells contributing to collagen proliferation, were observed in the interstitium. Regarding the breast cancer fibrosis or collagen proliferation caused by hormones, this phenomena is accelerated depending of immunological conditions (BRM) remarkably. SSM as BRM also promotes collagenation, so use of SSM for a long-term accelerate to produce further collagenation, resulting in cicatrization of tumor. Therefore SSM plays an important role in the cicatrization of breast cancer (Fig. 2).

SSM (Z-100) appears to have much greater cytotoxicity against malignant melanoma than against cancer cells. Therefore, a very high concentration of SSM (Z-100) can be used in the treatment of malignant melanoma for a long-term without side effects, so a high concentration of SSM with chemotherapeutic anticancer agents promotes cancer cell death and separation caused mainly by collagenation, and then the encapsulation, or confinement of cancer, that leads to cicatrization. As a result, it is possible that metastasis could be prevented.

The degree of collagenation was related to the concentration of SSM, and 200 μg/ml of high concentrated SSM (Z-100) showed effects on athymic and

thymic mice.

On the other hands, survival extension could be expected in human with positive tuberculin reaction by treatment with SSM and Z-100.

Newly it is well known that SSM or Z-100 is excellent effective for restoration of leukopeny induced by irradiation therapy of cancer bearing patients.

Sadahira and co-workers[42-44] isolated and cultured erythroblastic islands (EI) from the spleens of phlebotomized mice. The isolated EI were composed of surrounding erythroid cells and central stromal macrophages (Mϕ), which were identified by Forssman antigen. Erythropoietic activity on the Mϕ surface continued, although their processes had retracted. Some EI showed synchronized expansion of erythroblasts and others showed differentiation to reticulocytes. On the other hand the Mϕ secreted colony-stimulating activity during culture. It was infrequently observed that erythroid and myeloid population simultaneously expanded on a central Mϕ. These results indicate and suggest that stromal macrophages play an important part on hematopoiesis and stimulated stromal Mϕ by SSM or Z-100 is effective for proliferation and differentiation of hematopoietic cells and SSM is useful for prevention of side effects.

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