0–6% of all spinal metastases.[1] Ependymomas and neurinomas are dominant around the cauda equina.[4] Metastatic tumor from outside the central nervous system in this region is very rare and, to our knowledge, only 11 cases including the present case (eight males, three females) have been reported in detail in the English literature.[1,2,4,6,8–12,14] The ages of the patients averaged 59.3 years (44–84 years). Wippold et al.[15] reported that the mean age of the patients with neurinoma was 49.7 years and that with ependymoma was 38.3 years. Metastatic cauda equina tumors seem to appear later in life than neurinoma and ependymoma of this region.

The pathologies and primary lesions of the metastases to the cauda equina were adenocarcinoma[4,10,11] (one case from prostate, one case from ovarium, one case from endometrium), renal cell carcinoma[6,8,14] (four cases including the present case from kidney), squamous cell carcinoma[1] (one case from anus), undifferentiated carcinoma[2] (1 case from lung), nasopharyngeal carcinoma[12] (one case), and lymphosarcoma[9] (one case from mediastinum). These tumors metastasized to the cauda equina three to six years (mean 4.6 years) after surgery of the primary lesions. According to the Stark's series[13] with 131 metastatic spinal tumors, the lung (43 cases) and breast (37 cases) were the most common primary lesion sites, whereas metastases from the kidney occurred in only four cases. The kidney, however, seemed to be relatively a common primary lesion site of metastasis to the cauda equina. In four out of 11 cases, renal cell carcinomas were the primary malignancy. The prevalence of brain metastasis ranges from the kidney 5.7–9.7% in autopsy studies and 3.0–32% in clinical studies.[14]

The symptoms of cauda equina lesions are known to be non-specific.[3,15] Low back pain was the most common symptom, followed by sciatic pain, sensory disturbance, motor weakness, and bladder dysfunction.[3,5,7,15] Of 11 patients with metastatic tumors, four showed only low back pain with or without sciatica, and the remainder complained of multiple neurological symptoms. A remarkable feature of the clinical course of primary cauda equina lesions was the longstanding preoperative history with a mean time of years.[5,7] The preoperative histories of the metastatic tumors varied from two to 36 months (mean 12.6 months). Metastatic tumors seem to evolve symptoms more rapidly than primary cauda equina tumors.

Five routes have been hypothesized for metastatic intradural spinal tumor from outside the central nervous system:[11] (a) haematogenous, via the arterial system, (b) through the rich venous plexus, (c) through perineural lymphatics, (d) spread via subarachnoid space, and (e) seeding from the involved osseous structure to the cerebrospinal fluid through the dura mater.[6] Arterial embolism through the lung seems to explain the unusual metastasis to the cauda equina in our case. He had been diagnosed as having metastatic lung tumors prior to admission. Some authors have suggested venous embolism through the venous channels between the pelvis and the spinal cord. This could be the mechanism, though it is unlikely. Metastasis through the perineural lymphatics is also unlikely. Neuroradiological examinations including abdominal MRI, CT scan and radioisotope images revealed no local tumor recurrence and no metastasis to the intrapelvic or paraspinal organs. Other authors have proposed that seeding via the subarachnoid space formed the metastasis around the cauda equina as drop metastasis.[10,12] This seems unlikely because brain MRI showed no metastatic brain tumor and his surgical findings revealed no evidence of subarachnoid dissemination of the tumor. Despite a radiological work-up, we could not diagnose correctly before surgery. Fifty-seven percent of clear cell renal carcinomas demonstrate mutations of the Hippel-Lindau locus at 3p25. Globogangliosides acting as adhesion molecules have been shown to be in-

creased in renal cell carcinoma metastasis. A new approach directed towards the molecular biology of renal cell carcinoma metastasis may offer more options for the treatment of this condition.[8]

The tumor in our case was elastic hard, well demarcated and did not infiltrate into the subarachnoid space. It involved a single spinal nerve that fanned out over the surface of the tumor. We could remove the tumor easily after resecting the nerve proximal and distal to the tumor. These surgical findings are typical for nerve sheath tumors but are extremely exceptional for metastatic tumors. All of the metastatic cauda equina tumors seemed to have involved several spinal nerves or disseminated in the subarachnoid space[8–12] with the exception of Takahashi's case,[14] where the operative findings resembled those of our case. Both cases had renal cell carcinoma. The postoperative course of our patient was satisfactory. He noticed complete relief of his radicular pain. He also showed no sensory and motor disturbance after surgery. The tumor might have metastasized to one of the sensory roots, and the function of the affected root might have been compensated by the adjacent sensory roots during tumor growth.

CONCLUSION

We reported a rare case of the metastatic tumor of the cauda equina from the kidney. The radiographic and the operative findings of the tumor quite resembled that of a nerve sheath tumor. Only 10 cases with solitary metastasis to the cauda equina were reported in the English literature and we added the 11th case.

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Review Article

Interferon-β gene therapy for cancer: Basic research to clinical application

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Interferon-β gene therapy for cancer is the first such protocol developed in Japan. Here we describe the development process of our interferon-β gene therapy from basic research to clinical application. Interestingly, the biological and biochemical characteristics of interferon-β gene therapy through transfer of the interferon-β gene into tumor cells by means of cationic liposomes differed from those of conventional interferon-β protein therapy. Interferon-β gene transfer could induce apoptosis in interferon-β protein-resistant tumor cells, such as glioma, melanoma, and renal cell carcinoma. Induction of apoptosis was related to the level of intracellular mRNA of interferon-β, prolongation of the phosphorylation time of molecules in the interferon-β signal transduction pathway, such as JAK1, Trk2, and STAT1, and activation of DNase γ. In our preclinical study we developed lyophilized cationic liposomes containing interferon-β gene (gene drug) for clinical use and confirmed their safety. Thereafter, we performed a pilot clinical trial in patients with malignant glioma and confirmed the safety and effectiveness of this interferon-β gene therapy. In this review we also comment on the status of gene therapy regulation in Japan. Interferon-β gene therapy is expected to become widely available for clinical use in cancer patients, and this new strategy might be extended to molecular targeting therapy, or used in combination with cell therapy or other therapies. (Cancer Sci 2004; 95: 858–865)

S ince Watson and Click discovered the double-stranded structure of DNA in 1953, recombinant DNA technology and molecular biology have developed rapidly, and the complete sequence of the human genome, consisting of 3 billion base pairs, has been identified through the human genome project (1990–2003). This has opened the way for a new generation of advanced technology-based medicine, including gene medicine (genetic diagnosis and gene therapy), regenerative medicine, robotic medicine, molecular medicine, and nanomedicine. In particular, gene therapy offers tremendous promise for the future treatment of cancer.

The first gene therapy was initiated on September 14, 1990 in the USA for a patient with severe combined immunodeficiency (adenosine deaminase [ADA] deficiency). Since then, there have been more than 600 trials worldwide, and more than 4000 patients have received some kind of gene therapy. Recently, the target diseases have been extended from congenital metabolic disorders to malignant tumors which cannot be cured by existing treatments, and even chronic benign diseases which result in a poor quality of life.

In Japan, gene therapy for ADA deficiency began in 1995 at Hokkaido University Hospital using the same protocol as in the USA. To date, twenty gene therapy protocols have been developed. Among them, fifteen are related to cancers. Targeted diseases include renal cell carcinoma, lung cancer (non-small cell carcinoma), esophageal cancer, breast cancer, prostate cancer, brain tumor (malignant glioma), leukemia, and colon cancer. In the Department of Neurosurgery and Molecular Neurosurgery, Nagoya University Graduate School of Medicine, we have started a gene therapy protocol of our own since April 2000, an interferon (IFN)-β gene therapy using cationic liposomes. This protocol is the first using made-in-Japan technology. Here we summarize the developmental process of our human IFN-β gene therapy for patients with malignant glioma.

1. Basic studies

Malignant gliomas are too invasive to cure with surgical resection alone. In general, patients with malignant glioma undergo adjuvant therapy, including radiation therapy, chemotherapy, and immunological therapy after surgical resection, but their prognosis remains poor. Gene therapy is a much-awaited new strategy to overcome the misery associated with this neoplasm. However, only a few gene therapies have been directed toward diseases of the central nervous system (CNS), because the CNS is a very complex and critical organ, and access to it is restricted by the blood-brain barrier (BBB). It is therefore important to develop an appropriate delivery system. We currently use a liposomal gene delivery system.

1-1 Liposomes as a gene delivery system

Liposomes are artificial lipid bilayer vesicles considered to be useful as a drug delivery system. They can be morphologically divided into three types: small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV) (Fig. 1A). In the 1980s, it was well established that liposomes bearing positive charges on their surface could provide more efficient delivery of their entrapped components into cells, compared to other types of liposomes.[1,2] Our research on cationic liposomes was initiated in 1988. We found, in collaboration with Yagi and his colleagues, that cationic liposomes consisting of N-(α-trimethylammonioacetyl)didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE) (1:2:2 or 1:2:3, molar ratio) provide high-efficiency DNA entrapment and a high potential for DNA transfer to human glioma cells.[3–8] Moreover, DNA/liposomes are one of the safest delivery modalities to the CNS. Shin et al. reported that delivery of genes via DNA/liposome complexes to the brain could be achieved by incorporating antibodies to the transferrin receptor in order to facilitate passage across the BBB.[9]

Recently, various modifications of liposomes, e.g., combination of adeno-associated virus (AAV) vectors, adenovirus vectors, and nanotechnology-based molecules (Fig. 1B),[10–12] have been investigated.
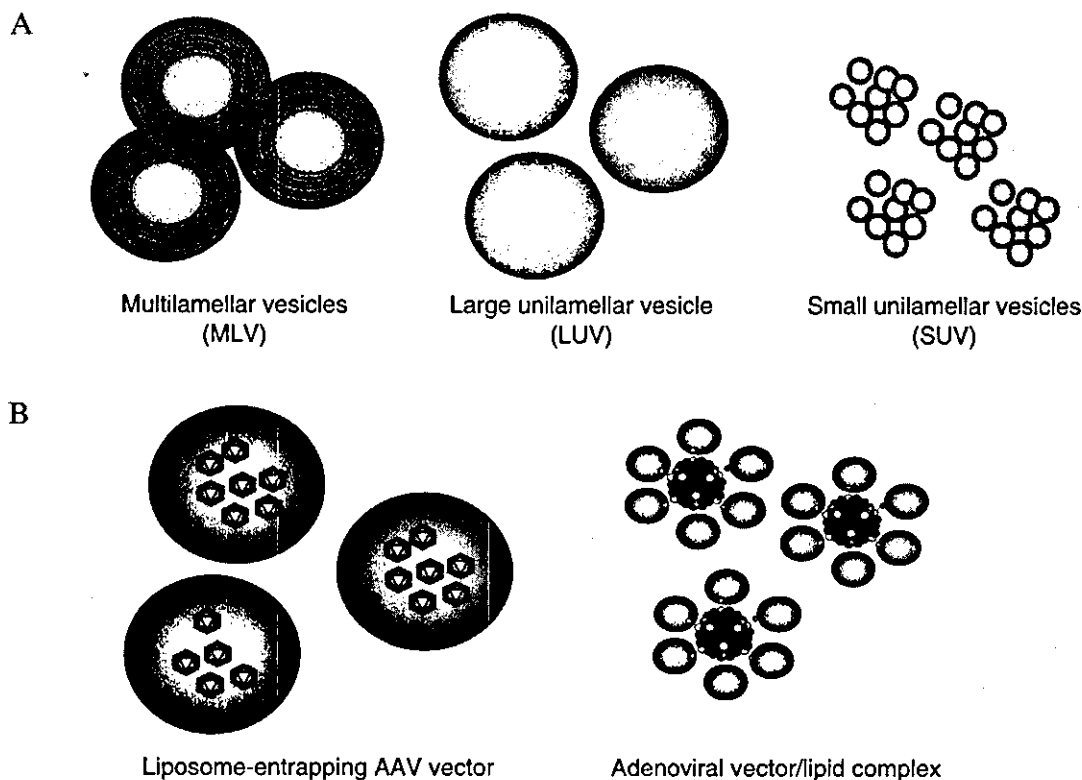
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Fig. 1. Cationic liposomes and their application. A. Cationic liposomes can be morphologically divided into three types: small unilamellar vesicles (SUVs); large unilamellar vesicles (LUVs); and multilamellar vesicles (MLVs). SUVs bind the genes to their surfaces, producing DNA-lipid complexes. SUVs have already been applied to clinical cancer gene therapy, especially immuno-gene therapy. LUVs and MLVs generally entrap the genes within the liposomes, rather than on the surfaces. MLVs can retain transfection efficacy for more than 1 year. B. Cationic liposomes have high potential for a variety of modifications. For example, they have a higher potential to combine with other materials such as viral vectors, antibodies, and other therapeutic agents. In our university, we are striving to develop combined therapy with liposomes and adeno-associated virus (AAV) vectors or adenovirus vectors. AAV vector-associated liposomes have more than 10-fold greater transduction efficiency than liposomes containing plasmid DNA and more than 6-fold greater than AAV vector alone. In contrast, adenoviral vector/lipid complex reduces the antigenicity of the adenoviral vector *in vivo* without diminishing the antitumor activity.

1-2 IFN-β

Human IFN-β is thought to be an important factor in the growth of human glioma and melanoma because homozygous deletions of the class I IFN gene cluster, comprising multiple IFN-α genes and a single IFN-β gene, have been demonstrated in these tumors.[13, 14] In the 1980s, IFN-β protein was clinically used as an anticancer drug in Japan, and it showed a clear growth-inhibitory effect on malignant glioma and melanoma.[15, 16] However, tumor regression was observed in only 10–30% and 15–20% of the patients treated for glioma and melanoma, respectively, and survival prolongation was not attained in either.[17] IFN-β protein also deserves attention as a cell-cycle regulator, inducing aberrant cell-cycle progression, which occurs predominantly as S phase accumulation, and less frequently as other cell-cycle effects, such as G1 arrest, or the entry of tumor cells into a senescent-like state.[18]

1-3 Antitumor mechanisms of IFN-β gene transfer

We investigated the antitumor activity of IFN-β gene therapy in both *in vitro* and *in vivo* experiments. In the *in vivo* experiments, human glioma cells were implanted into the brain of nude mice. One week after implantation, the tumor cells formed a mass 2 mm in diameter. From this time point, we started injection of liposomes containing human IFN-β gene 6 times every other day. One month later, the tumor was eradicated completely, although repeated direct injections of human IFN-β protein (1000 IU) did not suppress the tumor growth at all. We therefore analyzed in detail the mechanisms of this sur-

prising antitumor effect induced by IFN-β gene transfer. From our previous experiments, we speculated that the IFN-β gene has four main anti-tumor effects on glioma cells (Fig. 2). Interestingly, IFN-β gene transfer by means of cationic liposomes induces apoptosis of cultured human glioma cells that are resistant to IFN-β protein.

Fig. 3 summarizes the molecular mechanisms of apoptosis induced by IFN-β gene transfer via cationic liposomes. Susceptibility to extrinsically supplied IFN-β protein correlated closely with the amount of intracellular IFN-β mRNA in cultured human glioma cells, in agreement with the findings of Hanson *et al.* in melanoma cell lines.[19] It was also confirmed that there is a significant prolongation of phosphorylation time of several proteins involved in the intracellular signal transduction pathway of IFN-β, such as JAK1, Tyk2, and STAT-1. This apoptotic process did not involve caspase-3 or 8 activation and cleavage of DFF45/ICAD, but activation of caspase-7 and DNase γ was detected.[20] Besides the activation of DNase γ, the interaction of cell membranes and lipid membranes on cationic liposomes in IFN-β protein-resistant cells was also required (Fig. 3). Accordingly, cell death induced by the IFN-β gene delivered in cationic liposomes probably involves at least two elements, i.e., the intrinsic apoptotic pathway and mitotic catastrophe. The former is a pathway triggered by various extracellular and intracellular stresses, such as hypoxia and DNA damage. The stress signals converge mainly on mitochondria, forming the apoptosome which contains cytochrome *c*, apoptotic protease activating factor-1 (Apaf-1) and caspase 9, and
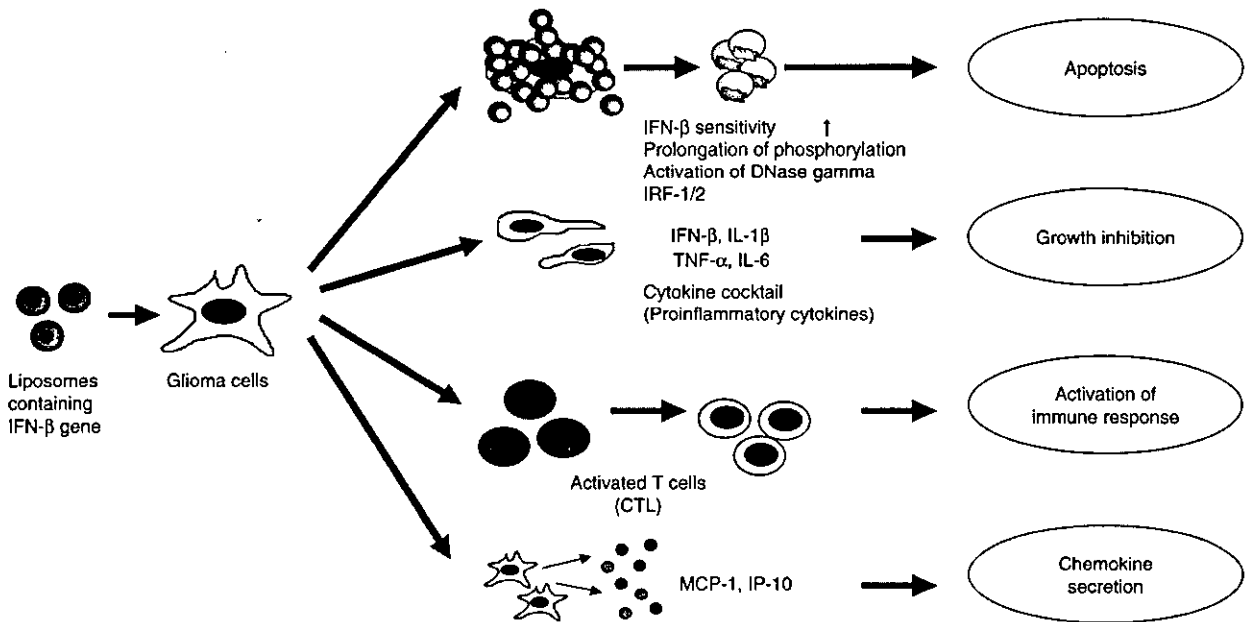
Fig. 2. Summary of antitumor mechanisms of IFN-β gene therapy for malignant glioma. There are at least four antitumor mechanisms of IFN-β gene therapy for malignant glioma; induction of apoptosis. IFN-β gene transfer by means of cationic liposomes can induce apoptosis in IFN-β protein-resistant cells; IFN-β gene transfer to glioma cells produces some cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in addition to IFN-β. The mixture of these cytokines exerted a strong antitumor effect on glioma cells; IFN-β gene transfer activates systemic immune responses and facilitates immune cell infiltration into a brain tumor, although the brain is an immunologically privileged site. Tumor-infiltrating cells are mainly CD8-positive cytotoxic T lymphocytes (CTLs) and macrophages; IFN-β gene transfer to glioma cells also produces some chemotactic factors such as monocyte chemotactic protein (MCP)-1 and IP-10.

leading to activation of caspase-7, followed by activation of DNase γ. The latter results in mitotic catastrophe, a pathway triggering mammalian cell death through aberrant mitosis. In fact, IFN-β gene transfer by means of cationic liposomes increased the number of multinucleated giant cells, i.e., the rate of abnormality of chromosome segregation, and subsequently induced apoptosis in cultured human glioma cells. Accordingly, we speculate that IFN-β gene transfer could be relevant to mitotic catastrophe in human glioma cells.

In addition to apoptosis, glioma cells transduced by the IFN-β gene produced interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, IFN-γ-inducible protein-10 (IP-10), and heat shock protein (HSP) in addition to IFN-β. The mixture of these cytokines exerted a strong antitumor effect on glioma cells. Duguay et al. reported similar results, namely that IFN regulatory factor (IRF)-3 gene transfer, which has been shown to activate type I IFN genes, can mediate important antitumor responses, increasing the inducibility of mRNAs for cytokines such as IFN-β, TNF-α, IL-6, MIP-1α, RANTES, and IP-10.[21] Moreover, IFN-β gene transfer activated systemic immune responses and facilitated immune cell infiltration of the brain tumor, despite the fact that the brain is an immunologically privileged site. Tumor infiltrating cells were mainly CD8-positive cytotoxic T lymphocytes (CTLs) and macrophages. According to Zhang et al., IFN-β gene therapy for human prostate cancer stimulated expression of inducible nitric oxide synthase, down-regulated transforming growth factor (TGF)-β and IL-8, reduced microvessel density, and resulted in apoptosis of endothelial cells in the lesions. These data suggest that macrophages may play an important role in IFN-β gene therapy.[22] Additionally, we found that dendritic cells (DCs) or macrophages which had been injected into the brain moved to the cervical lymph node. This finding suggests that macrophages may transport antigen information to the cervical lymph node. We also found that subcutaneous injection into the neck of plasmacytoid DCs, which are

CD11c(+) and B220(+), effectively promoted the infiltration of CTLs in a mouse experimental glioma, while subcutaneous injection into the neck of myeloid DCs, which are CD11c(+) and B220(−), only slightly promoted it. From these findings, we speculate that there may be an "immune circuit" for brain tumors, which can be activated by the IFN-β gene delivered in cationic liposomes, as shown in Fig. 4.

2. Pre-clinical studies

In pre-clinical studies, we investigated how to make cationic liposomes containing IFN-β gene for clinical use and also how to assess safety. We have conducted careful, repeated safety tests on the liposomal products (Table 1). We did not encounter any problems in animal studies using several species, including mice, rats, dogs, and monkeys. Animal studies revealed that cationic liposomes containing the IFN-β gene are most effective when administered by a regimen that will maintain a constant low concentration of IFN-β protein for a definite time in treated tumors, rather than a single bolus administration.

We have developed freeze-dried (lyophilized) cationic liposomes, which retain therapeutic activity for more than 1 year. The lyophilization technique has made it possible to transport this gene drug (cationic liposomes containing IFN-β gene) to other institutes collaborating in this work. In 2004, we sent our gene drug to Shinshu University Hospital to begin clinical research on IFN-β gene therapy for malignant melanoma.

3. Clinical studies
3-1 Regulation of gene therapy (Fig. 5)

The regulation of gene therapy is intended to ensure not only proper assessment of risks, but also a suitable safety margin. Currently, any clinical trial should follow Good Clinical Practice (GCP) in compliance with worldwide consolidated GCP guidelines.

In our hospital, a gene therapy committee was formed prior to our clinical study. As the first step, we selected candidates
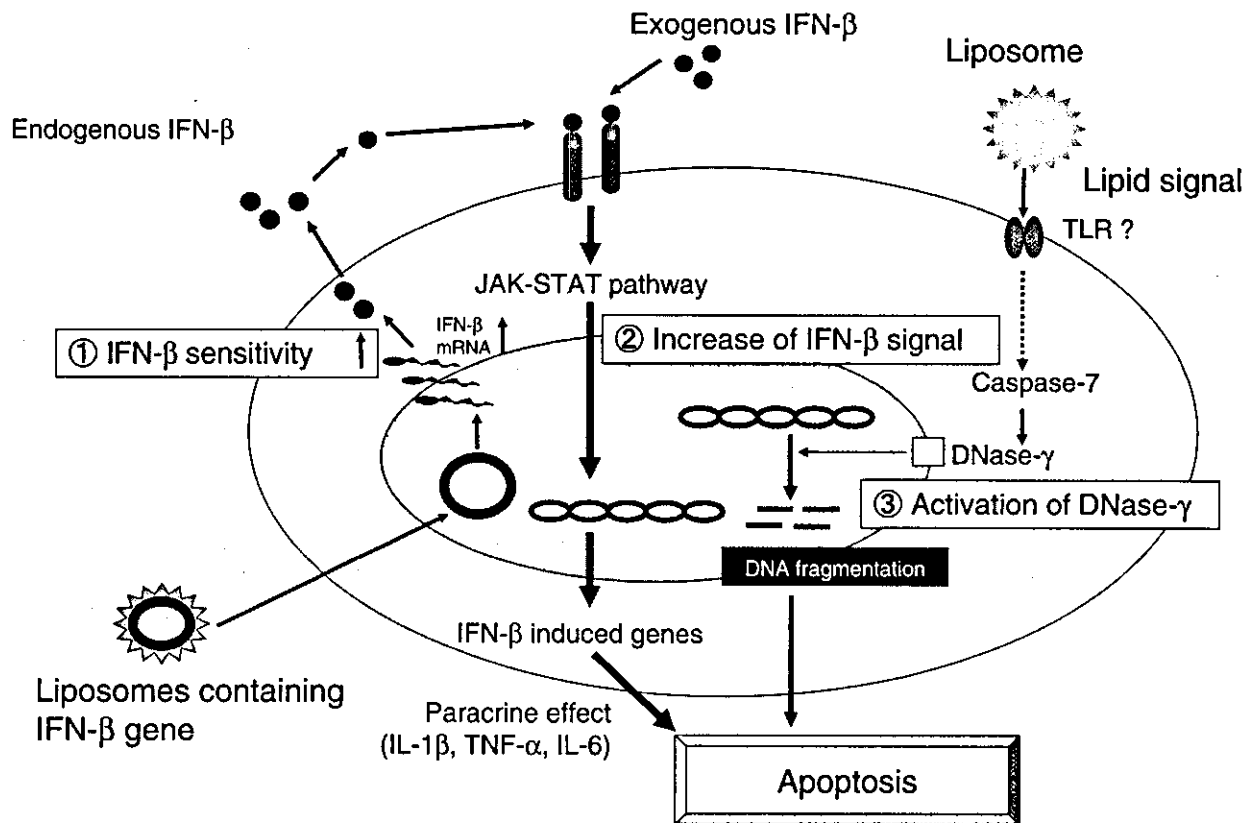
Fig. 3. Molecular mechanisms of apoptosis induced by IFN-β gene transfer by means of cationic liposomes. There are at least three molecular pathways of apoptosis induced by IFN-β gene transfer by means of cationic liposomes; the susceptibility to extrinsically supplied IFN-β protein correlates closely with the amount of intracellular IFN-β mRNA in cultured human glioma cells; IFN-β gene transfer causes a significant prolongation of phosphorylation time of several proteins involved in the intracellular signal transduction pathway of IFN-β, such as JAK1, Tyk2, and STAT-1; IFN-β gene transfer activates DNase γ through the interaction of cell membrane and lipid membrane on cationic liposomes even in IFN-β protein-resistant cells.
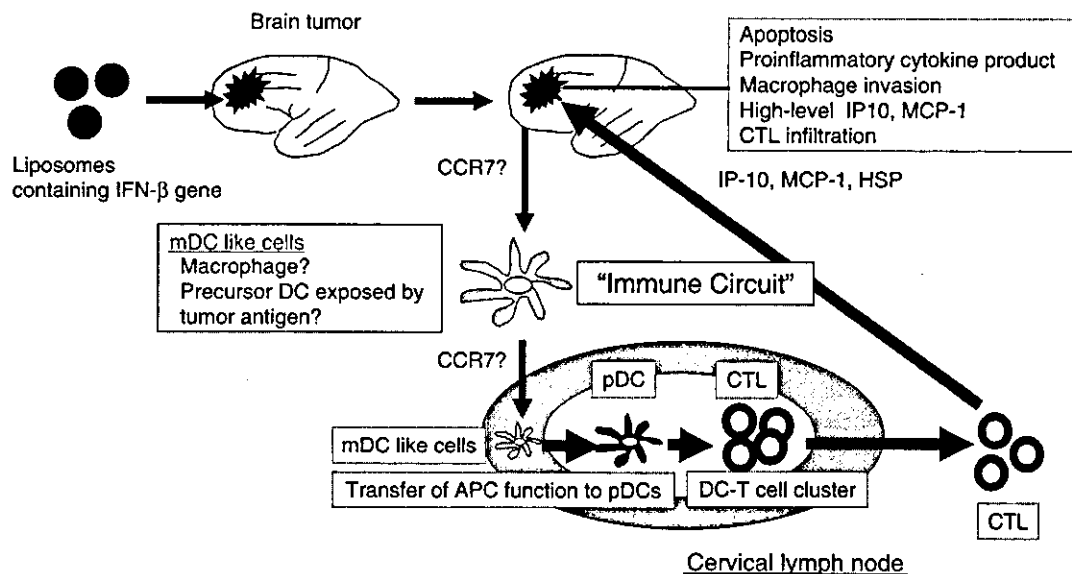

Fig. 4. Hypothetical pathway of cytotoxic T lymphocyte (CTL) activation in the brain induced by IFN-β gene therapy. We speculate that macrophages or plasmacytoid DCs (pDCs) probably transport antigen information to cervical lymph nodes, forming DC-T cell clusters, and then producing activated CTLs. The CTLs are guided to the brain tumor by IP-10, MCP-1, and HSP, then attack the tumor cells. mDC, myeloid dendritic cells; CCR, C-C chemokine receptor; APC, antigen-presenting cells; IP-10, IFN-γ-inducible protein-10; MCP-1, monocyte chemotactic protein-1; HSP, heat shock protein.

Clinical trials & development

GCP (ICH)
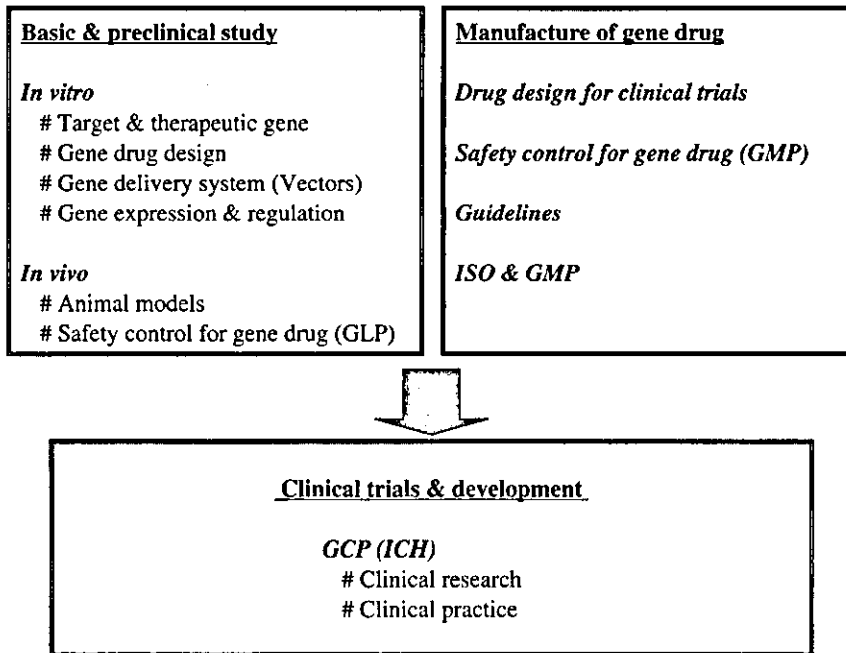# Clinical research
Clinical practice

Fig. 5. Regulation and implementation of gene therapy. GLP, good laboratory practice; GMP, good manufacturing practice; ISO, international organization for standardization; ICH, international conference on harmonization.

Table 1. Safety control for gene drug (liposomes containing human Interferon-β gene)

1. Single injection toxicity test
   (Intracranial and intravenous injection in rats and monkeys)
2. Repeated injection toxicity test
   (30-day repeat injection toxicity test for rats and monkeys)
3. Reproductive toxicity test
4. Deformity test
5. Antibody measurement
6. Toxicokinetics
7. Test for fever-producing activity in rabbits

No problems were detected in animal studies in several species including mice, rats, dogs, and monkeys.

who met the criteria for our gene therapy. Next, a subcommittee composed of several medical doctors, called the subcommittee for judging safety, efficacy, and indications, assessed the candidates using clinical data, and decided whether each patient would be a suitable candidate for our gene therapy. After a positive decision, a human gene therapy advisory board, composed of doctors, nurses, ethical specialists, legal professionals, and an outsider carefully reviewed the selection process again, to finally confirm the suitability of the candidate.

The manufacture and distribution of the gene drug were also of critical importance. Especially in the preparation of a gene drug, strict adherence to good manufacturing practice (GMP) is mandatory. In accordance with the regulations, our clinical-grade gene drug was produced in the Human Gene Therapy Vector Producing Facility at Nagoya University Hospital, where a fully documented quality management system is implemented. This system is similar to the management system of the International Organization for Standardization (ISO). In the near future, the development of advanced medicines will require ISO and GMP approval in Japan.

3-2 Clinical protocol

The patient received open surgery for tumor resection, followed by stereotactic injection of the gene drug. Treatment consists of reoperation and injection of liposomes containing human IFN-β gene on days 0, 14, 17, 21, 24, and 28 (first case)

or 0, 14, 21, and 28 (other cases). The surgical margin of the cavity after tumor removal was infiltrated with 1 ml of the gene drug at a concentration of 30 μg DNA/ml, evenly distributed at multiple sites. From injections 2 to 6, the procedure was repeated stereotactically under local anesthesia. After the 28th day of treatment, patients entered a follow-up period and were evaluated 3 months after the first injection, then every 3 months through the third year, and then annually until the study was terminated at the patients' death.

The clinical end points were evaluatation of the safety of this gene drug and determination of the efficacy of this gene therapy.

3-3 Case reports

So far we have performed the therapy on five patients. A brief summary is shown in Table 2. The tumor of patient 1 had shown rapid progression before gene therapy, with the volume increasing about 13-fold in only 4 weeks (3.0 ml to 38.7 ml). After gene therapy, tumor growth ceased, as measured by outlining the enhanced area in MRI, and there was little change in size for the following 10 weeks. Growth then resumed, and the patient died approximately 3 months later. Patients 2 and 3 each had a partial response (PR). Patient 4 could not be evaluated because no viable tumor cells could be confirmed to exist in the enhanced mass, after γ-knife therapy. Patient 5 had stable disease during the 10 weeks following the first injection. The general and neurologic condition of all patients was unchanged or improved 3 months after starting therapy, except for patient 5. Before the therapy, patients had various neurological deficits such as hemiparesis (patients 1 and 5), aphasia (patients 1 and 5), and memory disturbance (patients 2 and 4). In particular, patient 1 could not walk or speak before the therapy because of severe right hemiparesis and motor aphasia. However, at her discharge from the hospital 3 months later, she could walk by herself and talk with her family. Patients 2 and 3 had long survivals of 29 and 26 months after tumor recurrence, respectively.[23]

Transduced gene product in the fluid collected was assessed by EIA. Samples obtained from all patients before injection contained no detectable IFN-β protein. After injection, IFN-β protein was detected by EIA in fluid from the tumor bed in

cases 1, 2, and 5. The highest concentration, 23 IU/ml, was seen in patient 1 10 days after the first injection. IL-1β was detected in patients 1 and 5, and TNF-α was detected in patients 1, 2, and 5. Each protein was detected a few days after injection, reached maximum concentration 10 days later, then decreased gradually. IFN-β mRNA was also detected in tumor-rich tissues (patients 1, 2, 3, and 5) obtained by microdissection and examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). In contrast, mRNA for TNF-α was detected in tissues containing many macrophages (patients 1 and 2), but not in tumor-rich tissues. In addition, we confirmed the dramatic induction of immune response in the treated tumor tissues. After therapy, tumor tissues showed dramatic changes in all patients. Many tumor cells showed shrinkage or picnosis of the nuclei, reflecting apoptosis or necrosis. Simultaneously, MIB-1-positive cells were notably decreased. These alterations were observed over an area a few centimeters in diameter. Immunohistochemistry identified many CD8(+) lymphocytes and macrophages infiltrating into the tumor and

surrounding brain, while few CD4(+) lymphocytes or B lymphocytes were present.[23] These findings were the same as reported by Brown, i.e., the antitumor response mediated by IFN-β gene delivery relied on CD8(+) T cells, but was completely independent of CD4(+) T cells.[24] Notable cell infiltration was detected at 2 weeks after injection in all patients; infiltrates then gradually increased, persisting for at least 1 month after the first injection.

Based on these results, we compared the antitumor mechanisms observed in the basic and clinical studies. We confirmed that these were almost the same in all patients treated with IFN-β gene therapy, except patient 4 (Fig. 6).

4. Expansion of clinical indications to other malignancies
4-1 Melanoma

The incidence of malignant melanoma has been increasing by 5% per year for the last 40 years in Caucasian and other populations. Patients with this neoplasm have a poor prognosis. Unfortunately, there is no effective treatment when melanoma

Table 2. Clinical results of IFN-β gene therapy for recurrent malignant gliomas

Case	Gene therapy (pDRSV-IFN-β)	Results			
		Tumor size (MRI) (3 months after Tx)	Histology	TTP (month)	D (month)
1	30 μg×1 15 μg×5	Stable/progression	Tumor cell death Immune response	3	D (6)
2	30 μg×4	Partial response	Tumor cell death Immune response	16	D (29)
3	30 μg×1	Partial response	Tumor cell death Immune response	15	D (26)
4	30 μg×2	Stable	Tumor cell death Immune response	6	D (13)
5	30 μg×4	Stable	Tumor cell death Immune response	5	D (11)

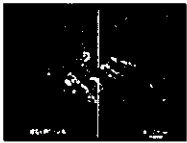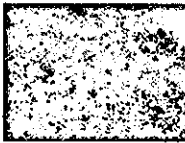TTP, time to pregression; D, dead; Tx, treatment.

Mechanisms	Basic Study	Clinical Study
Apoptosis	Apoptotic cells ↑	ApopTag-positive cells ↑
Cytokine mixture	IFN-β, IL-1β, IL-6, TNF-α	IFN-β, IL-1β, IL-6, TNF-α
CTL induction	CD8-positive cells in GL261 glioma ↑	CD8-positive cells in patient glioma ↑
Chemotactic factors & other	MCP-1, IP-10, HSP	MCP-1, IP-10

Fig. 6. Comparison of antitumor mechanisms of IFN-β gene therapy found in basic and clinical studies. Basic studies on the antitumor mechanisms of IFN-β gene therapy were consistent with clinical findings. HSP, heat shock protein.

is recurrent and/or at an advanced stage. Gene therapy has therefore received particular attention as a promising treatment modality for melanoma. Many investigators have performed new strategic gene therapies including IL-2, human leucocyte antigen (HLA)-B7β2m, granulocyte-macrophage colony-stimulating factor (GM-CSF), gp100, and MART-1. Recently, cationic liposomes have been used as a safer alternative to virus vectors in experimental and/or clinical trials on melanoma.[25, 26] We also confirmed that melanoma is susceptible to IFN-β protein, like glioma, and we assessed the growth-inhibitory effect of IFN-β gene transferred in cationic liposomes in *in vitro* and *in vivo* experiments. As expected, cationic liposome-mediated IFN-β gene therapy was effective against melanoma, inducing direct cell death and stimulating the host immune system.[27, 28] Thus, in experiments using an experimental human melanoma implanted subcutaneously in nude mice, we found extensive apoptotic tissue and a significant decrease of Ki67-positive cells after IFN-β gene transfer.[27] In experiments using subcutaneous mouse melanoma in immunocompetent mice, liposomes containing the murine IFN-β gene, but not recombinant murine IFN-β, induced dramatic apoptosis, including nuclear condensation, shrinkage of cells, bleb formation, and ballooning. Immunocytochemical analysis demonstrated that a larger number of natural killer cells infiltrated the tumor following the gene treatment as compared with the controls. *In vivo* depletion of NK cells using anti-asialoGM1 antibody reduced the efficacy of liposomes containing the murine IFN-β gene treatment. Taken together, our data demonstrated that cationic liposome-mediated IFN-β gene therapy could be effective against melanoma by inducing direct cell death and by stimulating NK cells.[28] In this experiment, we initially expected that IFN-β gene transfer would activate CTLs in melanoma as well as glioma. However, activated immune cells were not CTLs, but NK cells. Thereafter, it became clear that major histocompatibility complex (MHC) class I expression was small in the murine melanoma model (B16F1) used in this experiment. Moreover, we found that combined therapy of cationic liposome-mediated murine IFN-β gene therapy and DCs effectively induced CTLs because a co-culture of murine melanoma (B16F1), DCs, and naïve T lymphocytes produced large amounts of IFN-β and IL-12, and IFN-β increased MHC class I expression on the surface of the melanoma cells.

In any case, liposomes containing the murine IFN-β gene played an important role in activating immune responses. We found that DCs pulsed with tumor extract-cationic liposomes complex increased the induction of CTLs in mouse brain tumor.[29]

4-2 Renal cell carcinoma

We examined the feasibility of IFN-β gene therapy for renal cell carcinoma and confirmed that it was effective. Cationic liposomes containing IFN-β gene significantly induced apoptosis, although recombinant human IFN-β protein failed to do so, suggesting clinical applicability of gene therapy for renal cell carcinoma.[30]

5. Future directions

The success of gene therapy for cancer depends on a combination of applied bioengineering and so-called translational research. In the development of previous gene therapies for cancer, the biological principles were sound, but it proved difficult or impossible to translate these principles into reality. Although suitable systems with potential for cancer gene therapy have been known for a long time, efforts have generally remained at the preclinical stage, especially in Japan. In other countries, herpes simplex virus-thymidine kinase (HSV-tk)-based suicide gene therapy has just completed phase III trials. Therefore, our home-produced technology for IFN-β gene therapy is an important step forward.

Although the search for new vectors (viral and non-viral) continues, cationic liposomes are among the most fascinating vectors for cancer gene therapy because they are non-infective, have low immunogenicity, low toxicity, and high stability, and are not expensive to manufacture. The cost-benefit relationship is important, especially in the development of advanced medicines. Moreover, this protocol needs to be combined with other advanced medicines. Indeed, lessons learnt in developmental studies of cancer gene therapy may contribute to the development of other advanced medicines, such as molecular targeting therapy, regenerative medicine, cell therapy, and organ transplantation. The understanding of antitumor mechanisms in cancer gene therapy helps us to identify candidate target molecules for molecular targeting therapy and also leads to new approaches, such as the combination of gene therapy and chemotherapy or antibody therapy. The former approach is exemplified by the dramatic results with STI-571 (Gleevec) in chronic myelogenous leukemia, and the latter by the use of anti-vascular endothelial growth factor (Avastin) and anti-epidermal growth factor (Cituximab) monoclonal antibodies in the management of advanced colorectal cancer. An improved understanding of antitumor mechanisms in cancer gene therapy will have many spin-offs.

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Practice of Interferon Therapy
—Brain tumor—

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Interferon Therapy for Hairy Cell Leukemia

HCL is characterized by the development of cells with many hairy cytoplasmic projections. Since it becomes chronic, it is classified as a chronic leukemia. Typical hairy cells in Western countries are positive for tartrate-resistant acid phosphatase staining. There is a subtype called "Japanese type HCL", and its hairy cells show only weak tartrate-resistant acid phosphatase staining. Both types of HCL often cause splenomegaly.

HCL is treated with IFN therapy, splenectomy, and purine analogues. Recently, the efficacy of anti-CD20 antibody, rituximab, has been reported. The standard therapy for HCL should be started with splenectomy and, if the disease progresses, treatment with IFN or purine analogues should be considered.

The IFN therapy for HCL is based on the administration of 3 to 5 million units/day of IFN-α consecutively 3 times a week. The response rate of this therapy ranges from 50 to 90%.[6] Treatment with IFN improves blood cell abnormalities and reduces splenomegaly. Smaller doses of 0.2 to 0.6 million units of IFN will reduce the adverse effects, but will also reduce the efficacy rate. It is generally considered that the efficacy of IFN therapy for

Japanese type HCL is lower than that for European/American type.

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Practice of Interferon Therapy
—Brain tumor—

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Abstract: This paper outlines the current clinical application of interferon-β for treating brain tumor. Since approved as a therapeutic drug for brain tumor, IFN-β has been reported to be effective when it was used alone, in combination with chemo-radiation therapy (IAR therapy), and as a maintenance therapy. Recently, the regimens with IFN-β have been improved to obtain a higher efficacy rate. For example, liposome is used as a drug delivery system (DDS) to administer IFN-β protein or genes. Although much remains to be examined about administration methods for DDS, it is expected that new developments in the field of gene therapy will improve the therapeutic results of antitumor therapies by cytokines including interferon.

Key words: Brain tumor; Interferon-β; IAR therapy; Gene therapy

Introduction

Interferon (IFN) was discovered in the 1950's during research on viral interference, and its antitumor and other effects were reported from the 1960's. During the 1970's, attention was paid to IFN as an anti-cancer drug because its anti-tumor effect was reported in clinical studies. Now, it is clinically used to treat Type C hepatitis, multiple sclerosis, and various tumors including renal tumor, malignant melanoma, and brain tumor.

IFN is classified by its properties into 3 types:

IFN-α, IFN-β, and IFN-γ. IFN-α and IFN-β code common gene loci and have common cellular surface receptors, while IFN-γ has different dynamics. Therefore, the former and latter are called Types I and II IFN, respectively. In the clinical application of IFN for brain tumor, IFN-α is mainly used in Western countries, while IFN-β was approved by the Ministry of Health, Labor, and Welfare and has been clinically used in Japan.

This paper describes the history of the clinical application of different types of IFN for brain tumor, current issues, and prospects for

new therapeutic techniques.

Interferon Single Therapy

In studies of the antitumor effect of IFN-α and IFN-β using brain tumor cells, Lundblad[1] and Wakabayashi[2] reported a direct inhibitory effect on brain tumor, and Otsuka et al.[3] reported an indirect inhibitory effect through immuno-competent cells. Nakagawa and Ueda reported the clinical effect of IFN-α on malignant brain tumor: they achieved partial response in patients with glioblastoma and medulloblastoma by the systemic and local administration, respectively, of IFN-α. Subsequent phase II studies on the use of recombinant IFN-α for malignant glioma showed a response rate of 10.3 to 20%.

Nagai et al., who performed the systemic and local administration of IFN-β, reported an overall response rate of 22.2% in 54 evaluable patients, and the response rates of 16.7% and 42.9% in patients with glioblastoma and medulloblastoma, respectively.[4] In 10 patients with malignant glioma, Yoshida et al. systemically administered 3×10^5 to 3×10^6 units of IFN-β for 16 to 50 days continuously via an intravenous route or locally administered 5×10^4 to 3×10^6 units into the tumors for 7 to 73 days continuously via an Ommaya reservoir implanted when a tumor was removed. The result showed the size of the tumor was reduced by 50% or higher in 2 of 7 systemically treated patients and 1 of 3 locally treated patients.

However, it was reported that, in any case, the antitumor effect after the administration period lasted for only a short period, and that the administration of IFN alone would not eventually prolong survival, although it might provide remission during the administration period. Therefore, investigators started to attempt various regimens with IFN.

Interferon Combination Therapy

To improve the therapeutic results of the interferon single therapies for brain tumor,

combinations with other therapies or drugs were attempted. So far, the following combinations have been examined.

1. Combination with radiotherapy

For the combination with radiotherapy, which has been the most effective adjuvant therapy for malignant brain tumor, Miyoshi et al.[5] and Korosue et al.[6] performed basic research with IFN-α and IFN-β, respectively. The following hypotheses were obtained: partially synchronized radiotherapy with IFN in relation to the DNA synthesis inhibiting effect of IFN might be effective; IFN might play a role by sensitizing patients to radiation; and there might be an interaction between sublethal damage by radiation and the direct antitumor effect of IFN.

Regarding clinical applications, Mahaley et al. reported that the combination of radiotherapy and IFN significantly prolonged the median survival time in patients with malignant glioma, and that the combination provided better results than the combination of radiotherapy and BCNU (carmustine), which was previously the standard therapy for brain tumor patients in the institution.[7]

2. Combination with chemotherapy

Various combinations of IFN and anticancer agents have been examined. The Mayo Clinic reported that the combination of BCNU and IFN-α caused a significantly higher synergistic effect than that with other drugs in 35 patients with recurrent glioma: the combination achieved an efficacy rate of 29% and a period of 10.1 months, and blocked the progression of the disease for 6 months or longer in 37% of the patients. Nitrosourea anticancer drugs, such as ACNU (nimustine hydrochloride) and MCNU (ranimustine), are available in Japan, but single therapy with any of the drugs has been effective for only 30 to 50% of patients with brain tumor.

Examination of the combination of IFN-β and ACNU with 13 human glioma cell lines showed the combination 5 mg of ACNU and

1×10^3 IU of IFN-β provided a tumor prolifera-tion inhibiting effect of at least 2 log cell kill, and that the effect was obtained in 9 cell lines, as compared with 2 and 1 cell line by the single therapy with ACNU and IFN, respectively. Fur-ther, the effect was higher than that of at least 2 log cell kill observed in 7 cell lines treated with 10 mg of ACNU alone.[8] When ACNU is clinically applied at a usual dose of 2 to 3 mg/kg body weight, the concentration obtained in brain tumors is approximately 1 to 5 mg. It is practically impossible to increase the dose because of possible adverse effects, such as bone marrow suppression. Therefore, the re-sults indicating the potentiation of the antitu-mor effect more than the addition of the effect of each anticancer drug and IFN at a usual dose suggest the effectiveness of the combination therapy.

3. Combination with radio-chemotherapy

Since the combination of IFN-β and ACNU showed high antitumor activity in a basic ex-perimental study with a human glioma cell line, a clinical study was started in Japan by combin-ing the IFN-β and ACNU combination therapy with radiotherapy (IFN-β-ACNU-Radiation [IAR] therapy) as an adjuvant therapy for malignant glioma. Yoshida et al. reported that the prognosis as determined by the mean sur-vival period was significantly improved with IAR therapy (25.3 months) as compared with radiation alone (15.2 months) and radiation + ACNU (19.7 months), and that the initial re-sponse rate by IAR therapy was higher than that by radiation + ACNU (60.5% vs. 35.7%). Further, Yoshida et al. also confirmed the effi-cacy of IAR therapy in 175 malignant glioma patients followed for a long time.[9] Hatano et al. reported that increasing the administration frequency of IFN-β to twice daily increased its antitumor effect.[10] A U.S. study on the combination of IFN-α, BCNU, and radiation for malignant brain tumor reported a median survival time of 12.7 months and a mean sur-vival time of 16.1 months for Grade IV astro-
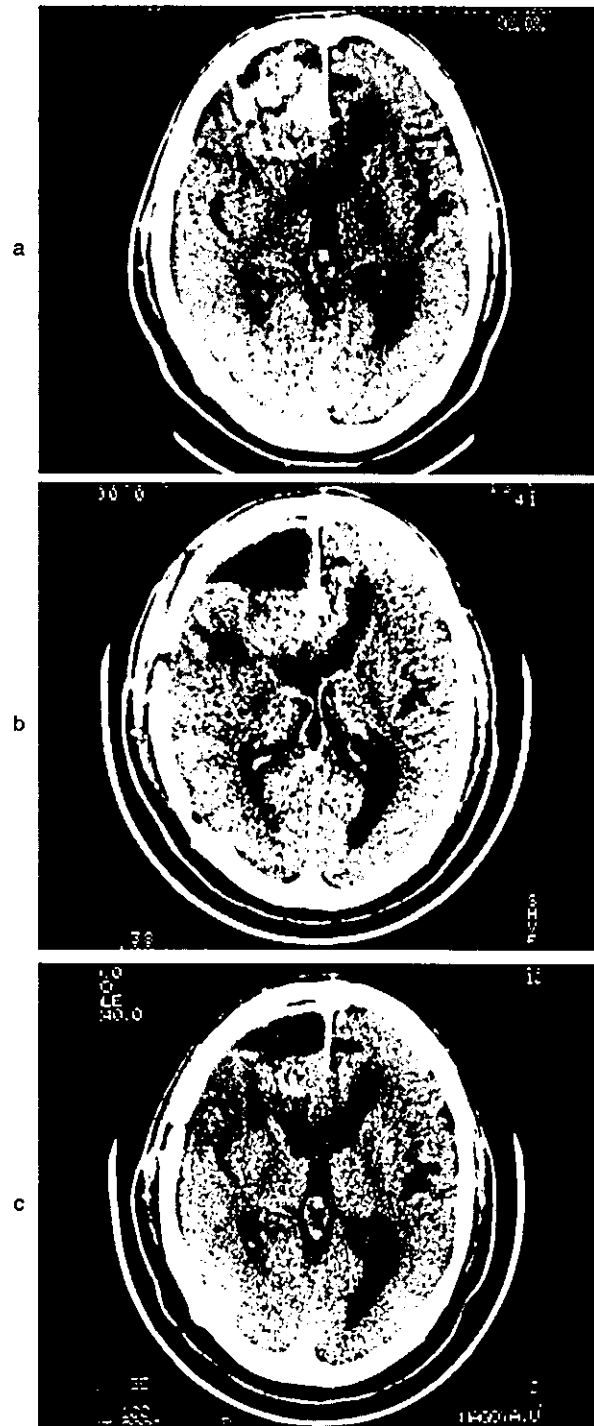

Fig. 1　54-year-old male patient: An enhanced lesion on CT scan indicating a tumor was observed in the right frontal lobe (a). It was diagnosed as glioblastoma. Since the postoperative image showed a residual tumor (b), IMR therapy was performed. A marked reduction of the tumor was observed at the end of the initial induction therapy (c). This patient received 3-month maintenance therapy, with no tumor recur-rence for the subsequent 2 years.

cytoma, and 46.3 and 61.3 months for Grade III astrocytoma.

These results indicate that the inter-disciplinary combination of IFN-β, nitrosourea drug, and radiotherapy should be the first-line initial adjuvant therapy for inducing remission after an operation for malignant brain tumor (Figs. 1-a, b, and c). However, since recurrence was observed in most of the cases who responded to the combination, it is necessary to establish an appropriate maintenance therapy at an early stage after the induction of initial remission.[1]

Interferon Maintenance Therapy

Although up to 60% of patients with malignant brain tumor could achieve remission by initial induction therapy, most of them experienced recurrence. For example, the remission and mean survival periods of patients with glioblastoma were reported to be as short as 11.2 and 13.9 months, respectively. Therefore, various maintenance therapies following initial induction therapy are being examined.

Wakabayashi et al. performed IFN-β-MCNU-Radiation (IMR) therapy as an initial induction therapy in patients who developed malignant glioma for the first time, and compared the remission period between those treated with a maintenance therapy consisting of 1×10^6 units of IFN-β every 2 weeks and 80mg/m^2 of MCNU every 6 weeks for at least 3 months after the end of the induction therapy and those not treated with it. The patients registered into the initial induction therapy were randomly divided into 2 groups with and without the maintenance therapy, and they were compared for time to tumor progression (TTP) and total survival period. The results showed a significantly prolonged survival period in the maintenance therapy group (Fig. 2). Particularly, the patients who achieved complete remission by the initial induction therapy appeared to achieve a significantly prolonged remission period by receiving the maintenance therapy. It was also suggested that a certain
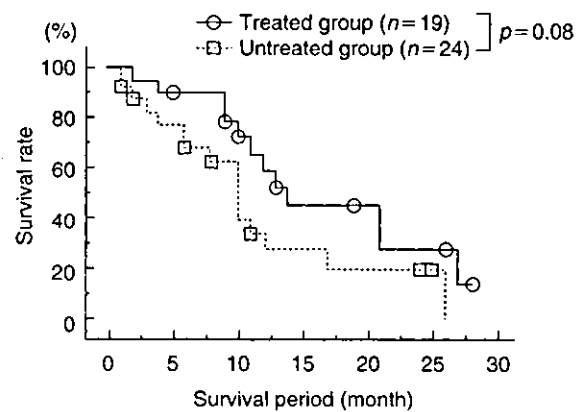

Fig. 2   Comparison between groups treated with and without IMR maintenance therapy

therapeutic effect could be expected from the maintenance therapy in patients who developed the disease for the first time at 47 years or younger, or who achieved a partial response or a better response with the initial induction therapy. These results suggest that both initial induction and maintenance therapies may be important for the treatment of malignant glioma.[12]

New Developments in Interferon Therapy

1. Drug delivery system (DDS)-combined interferon (liposome)

Although IFN-β has been clinically applied for treating brain tumor, the clinical efficacy of IFN-β alone is less than expected when it was introduced in an uncombined form. It seems necessary to combine it with other therapies or drugs to fully realize its potential. In fact, IFN-β shows a marked antitumor effect at as low as 100 units in vitro, while it has been reported to produce tumor reduction by 50% or higher only in 15% of clinical cases, even at 10 million units. This difference in the efficacy of IFN may be explained by the pharmacokinetics of IFN, its stability in blood or tissue, or the blood brain barrier.

In an effort to overcome the problem of the

low *in vivo* effect of IFN, liposome has been examined as a drug delivery system (DDS). Epstein *et al.* examined the embedding of IFN in liposome and successfully changed the biological activities and pharmacokinetics of IFN. Kato *et al.* added sulfatide to liposome as a component to deliver IFN through the blood brain barrier into the cerebral parenchyma, and compared the stability, pharmacokinetics, intraorgan distribution, and antitumor effect between the embedded and free IFN. The result showed the blood titer of the free IFN became undetectable as early as 2 hours after intravenous administration, while the liposome-embedded IFN was detected at as high as $10^3 \text{IU}/\text{m}l$ or higher even 8 hours after administration. Further, an IFN titer of $100 \text{IU}/\text{g}$ tissue or higher was confirmed in the brain and subcutaneously implanted brain tumor tissue where no IFN was detected after the intravenous administration of the free IFN. It is expected that the clinical application of DDS will progress to increase the effectiveness of IFN for brain tumor.[13]

2. Interferon gene therapy

Larsson *et al.* reported that endogenous IFN-β was produced from glioma cells using a super-induction technique. This glioma-derived endogenous IFN-β has an antitumor effect on human glioma cells. We have been developing IFN gene therapy in which human IFN-β genes are embedded in the liposome with an affinity for glioma cells to selectively introduce the liposome into glioma cells and locally generate a large amount of endogenous IFN-β, thereby causing an antitumor effect on glioma. Since this technique ensures the secretion and maintenance of a much higher local concentration of IFN than administration from outside, the so-called paracrine effect can be expected. Further, the technique has been reported to cause phenomena that have not been observed with exogenous IFN, such as the induction of apoptosis of transgenic glioma cells. Finally, we expect an association with the immune system

to indirectly enhance the antitumor effect.[14]

A clinical study on the gene therapy for brain tumor (malignant glioma) using this positively-charged liposome embedded-human IFN-β gene (local injection of the IFN-β gene-embedding liposome into brain tumor) was started on April 3, 2000 at the Nagoya University Hospital. So far, 5 patients have been registered and examined for the safety and efficacy of the therapy. The results of the study will be reported soon.

Conclusions

This paper outlines the current clinical application of interferon to brain tumor. There remains much to be examined about the use of IFN, such as appropriate administration regimens and the importance of maintenance therapy. However, together with the new developments in IFN therapy including the use of DDS and gene therapy, it is expected that the therapeutic results of antitumor therapies with cytokines including IFN will be improved.

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THE COMBINATION OF MITOTIC AND KI-67 INDICES AS A USEFUL METHOD FOR PREDICTING SHORT-TERM RECURRENCE OF MENINGIOMAS

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BACKGROUND
The most relevant factor in the progression-free survival (PFS) of patients with meningiomas is the malignant grade. However, using only the current World Health Organization (WHO) definition that does not consider precise quantitative indicators, an unequivocal diagnosis of the malignant grade is difficult. In our retrospective study of the PFS of meningioma patients, we focused on mitoses and the Ki-67 staining index of tumor specimens obtained at the initial surgery.

METHODS AND RESULTS
A total of 349 patients with intracranial meningioma, operated between 1978 and 2000, were followed for a mean of 7 years. According to the mitotic index (MI), we classified them into 3 groups. In Group A (n = 326), slide-mounted tumor samples exhibited no mitoses; in Group B (n = 15) there were fewer than 4 mitoses, and in Group C (n = 8) 4 or more mitoses were seen per 10 high-power fields (HPF). The estimated 5-year PFS rates in Groups A, B, and C were 93%, 10%, and 13% respectively. The mean PFS for Group A was 148 months; in Groups B and C the median PFS was 43 and 16 months, respectively. A Ki-67 staining index (SI) of less than 1% corresponded with no mitosis, while an SI exceeding 5% was indicative of the presence of mitoses.

CONCLUSION
In meningioma patients, no mitoses and/or a Ki-67 SI < 1% signals a favorable outcome. An SI >5% or the presence of mitoses, even fewer than 4 in 10 HPF, is suggestive of a short PFS irrespective of other pathologic features. We suggest that in combination, assay of the Ki-67 SI and the MI represents a reliable, quantitative tool for predicting PFS in meningioma patients. © 2004 Elsevier Inc. All rights reserved.

A lthough meningiomas are generally slow-growing, benign tumors, 20% of gross-totally resected meningiomas recur within 20 years [14]. The most relevant factor for progression-free survival (PFS) is their histologic malignant grade: while approximately 7 to 20% of benign meningiomas recur, for atypical- and anaplastic meningiomas the recurrence rates are much higher at 29 to 40% and 50 to 78%, respectively [7,9,11,14,16,24,25]. Thus, while the value of histologic grading is obvious, their objective staging has been hampered by the fact that the World Health Organization (WHO) classification of meningiomas relies heavily on qualitative criteria without taking into account more precise quantitative indicators such as numerical scoring systems.

The mitotic index (MI) is only one numerical criterion of pathologic grades in the WHO classification of meningiomas [14]. For the purpose of diagnosing atypical and anaplastic meningiomas, increased mitotic activity is defined as the presence of 4 or more and 20 or more mitoses per 10 high-power fields (HPF), respectively. Proliferation indices as well as the MI are significantly correlated with tumor-doubling time [14,20] and a Ki-67 staining index (SI) of more than 5 to 10% reflects an increased risk of recurrence as well as a high grade of malignancy [14]. In typical cases with obvious pathologic features of malignancy, increased mitotic activity, and a Ki-67 SI far exceeding the cut-off value, an unfavorable outcome is easily predicted at the time of the initial surgery. However, the prognosis varies in patients with meningiomas that lack

the typical malignant features and exhibit few mitoses and a Ki-67 SI near the cut-off level.

In this retrospective study of 349 patients with surgically removed intracranial meningiomas, we assessed the correlation between the MI and the clinical outcome represented by their PFS. We paid special attention to the prognostic significance of the presence of any mitotic figures in tumor tissue samples obtained at the time of the initial operation. As we detected a relationship between the Ki-67 SI and the presence of even fewer than 4 mitoses/10 HPF, we now suggest that in combination, these two parameters represent a powerful and quantitative tool for predicting PFS even in some meningiomas that are not easily classified into a benign or atypical type according to the WHO criteria.

Subjects and Methods

PATIENT POPULATION

Between 1978 and 2000, 426 patients with intracranial meningiomas underwent surgical treatment at Kyoto University Hospital. Their medical records were carefully reviewed and all clinical, laboratory, radiographic, pathologic, and follow-up data were retrieved. Of the 426 patients, 349 were entered into this study because they fulfilled all of the following criteria: 1) they had undergone no previous surgical treatment of their tumors, 2) their postoperative follow-up exceeded 1 year, and 3) they did not have meningeal hemangiopericytoma. Before tumor recurrence, none of the 349 patients had received radiation therapy.

There were 118 males and 231 females; their mean age was 54 years (range 18 to 78 years). Diagnosis was based on hematoxylin-eosin (H&E) staining of tumor samples from the initial surgery; of the 349 meningiomas, 331 (94.8%) were benign, 16 (4.6%) were atypical, and 2 (0.6%) were anaplastic. According to WHO criteria, we made a diagnosis of atypical or anaplastic type based on whether the tumor samples manifested a MI of 4 or more or 20 or more mitoses per 10 high-power fields (HPF), respectively, as well as pathologic findings such as increased cellularity, small cells with a high nucleus: cytoplasm ratio, prominent nucleoli, uninterrupted patternless or sheet-like growth, and foci of necrosis [14].

We used the system of Simpson [28] to record the extent of tumor removal. Accordingly, 118 patients (33.8%) had undergone Grade I resection (complete with excision of the dural insertion), 150 (43.0%) Grade II (complete with coagulation of the dural insertion), 53 (15.2%) Grade III (incomplete, leaving

behind a small amount of tumor tissue), and 28 (8.0%) Grade IV (incomplete, with a large amount of residual tumor tissue). Of the 326 Group A patients, 110 (33.7%) had undergone Simpson's grade I resection, 146 (44.8%) Grade II, 46 (14.1%) Grade III, and 24 (7.4%) Grade IV. Of the 15 Group B patients, 5 (33.3%) had received Grade I resection, 2 (13.3%) Grade II, 4 (26.7%) Grade III, and 4 (26.7%) Grade IV. Of the 8 Group C patients, 3 (37.5%) had undergone Grade I resection, 2 (25%) Grade II, 3 (37.5%) Grade III, and none Grade IV. The extent of resection was deduced from the recorded description of the operation and confirmed by inspecting postoperative computed tomography (CT) and/or magnetic resonance (MR) images in the patients' medical records. Other clinicoradiological findings such as perifocal edema, tumor staining, feeding from pial arteries, tumor hardness, arachnoid penetration, tumor demarcation, and tumor location were also confirmed by inspection of the surgical records and/or neuroradiological images.

During a mean follow-up time of 83 months (range 12–253 months), 42 of the 349 patients (12.0%) experienced recurrence; of these, 31 (73.8%) had benign- and the remaining 11 had atypical- or anaplastic meningiomas. The criteria for diagnosing recurrence were the demonstration, during regular follow-up, MR and/or CT evidence of tumor appearance after complete resection, or of regrowth of residual tumors.

ASSESSMENT OF THE MI AND THE KI-67 SI

In all 349 patients, tumor tissues obtained at the first operation were subjected to MI determination. The specimens were fixed with formalin, embedded in paraffin, and tissue samples mounted on slides were stained with H&E. The MI was determined by counting the number of unequivocal mitotic figures in 10 consecutive HPF ($\times$ 400) containing the highest number of mitoses [25].

In 29 of 42 patients with tumor recurrence, the Ki-67 SI of tumor tissues obtained at the first operation was additionally determined. Tissue sections (4 μm in thickness) were deparaffinized and immunostained using the Ventana NX automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ). Briefly, the slides were heated in a 750W microwave oven (5 min $\times$ 4) in citrate buffer (pH 6) and then incubated for 1 hour with anti-Ki-67/MIB-1 (Dako, Carpenteria, CA) diluted 1:100 in bovine serum albumin/TBS. Immunoreactions were then performed according to the LSAB method using streptavidin-biotin complex (ABC)-labeled horseradish peroxidase and diaminobenzidine

(DAB). All tissue sections were examined at high-power magnification ($\times$ 400). The number of cells stained positively with anti-Ki-67 antibody and the total number of tumor cells were counted in several representative fields containing more than 1000 cells and their ratio was expressed as the Ki-67 SI (%) [20]. In areas with heterogeneous distribution of Ki-67 immunopositive cells, the area containing the largest number of Ki-67-stained cells was considered to represent the proliferative activity of the tumor.

Using the H&E-stained sections, 2 pathologists (NK, YN) independently made the histologic diagnosis and graded the tumors. They inspected H&E-stained or immunostained sections together, using a multi-head microscope, and assigned the MI and Ki-67 SI by consensus. Although they were cognizant of the diagnosis, they made their assessments before accessing any information pertaining to the outcomes.

STATISTICAL ANALYSIS

The relationship between the MI and the Ki-67 SI was examined by the Pearson correlation coefficient. In addition, PFS curves were prepared using the Kaplan-Meier method. The log-rank test was employed to determine whether the MI-based classification, the extent of surgical resection, the pathologic diagnosis, and other clinicoradiological parameters were associated in a univariate manner with PFS. Differences were considered statistically significant at $p < 0.05$.

RESULTS

CLINICAL OUTCOMES AND MI

The 349 meningioma patients were divided into 3 groups according to their MI. Group A (no mitoses) consisted of 326 patients (322 benign-, 4 atypical meningiomas), Group B (more than 0 and fewer than 4 mitotic figures/10 HPF) of 15 (9 benign, 6 atypical or anaplastic), and Group C (4 or more mitotic figures/10 HPF) of 8 patients with atypical or anaplastic meningioma. There were no cases with 20 or more mitotic figures per 10 HPF.

The 349 patients were followed for a mean of 83 months. As shown in Figure 1A, the estimated 5-year PFS rates in Groups A, B, and C were 93%, 10%, and 13%, respectively. The mean PFS for Group A was 148 months; in Groups B and C the median PFS was 43 and 16 months, respectively. By the log-rank analysis, the difference in PFS between Group A and the other 2 groups was significant (Group A vs. B, $p < 0.0001$; A vs. C, $p < 0.0001$; A vs.
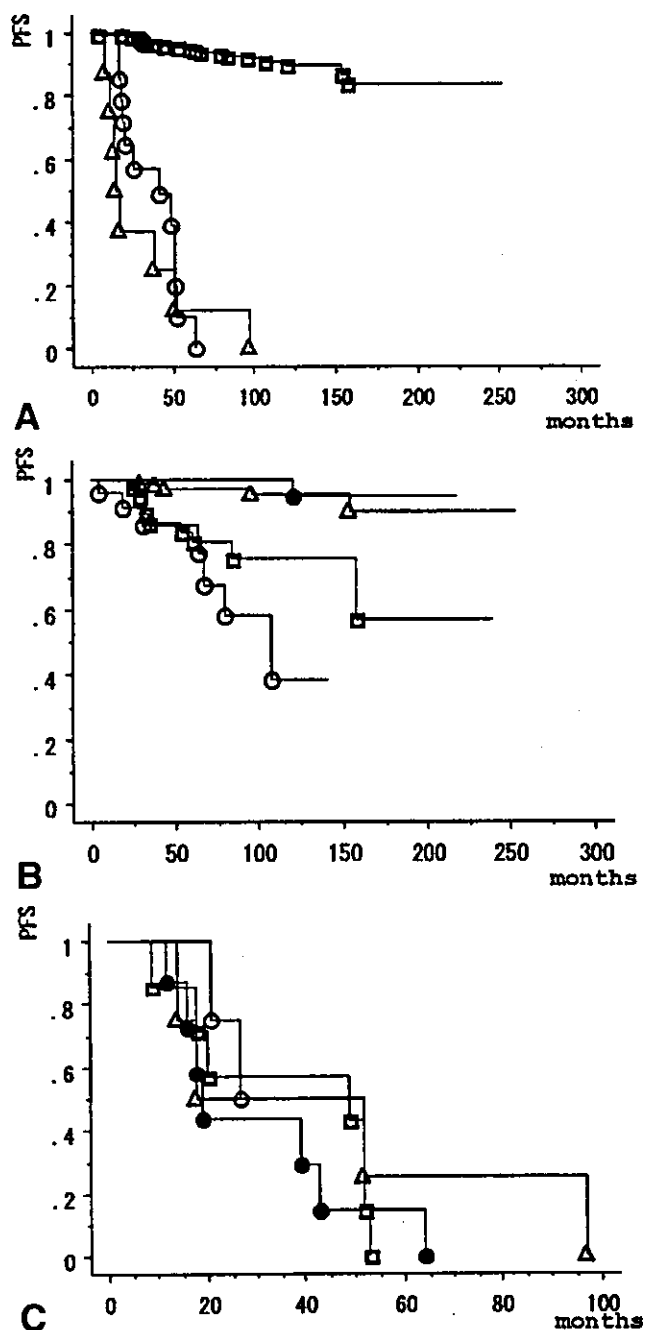
(A) Kaplan-Meier curves of progression-free survival (PFS) in patients grouped according to the mitotic index (MI) of surgical tumor specimens. Open squares, Group A (no mitotic figures); open circles, Group B (more than 0 and fewer than 4 mitotic figures per 10HPF); open triangles, Group C (more than 4 mitotic figures per 10HPF). (B) Kaplan-Meier curves of PFS in Group A patients classified according to the Simpson resection grade. Solid circles, Grade I; open triangles, Grade II; open squares, Grade III; open circles, Grade IV. For a description of Simpson grades, see Subjects and Methods. (C) Kaplan-Meier curves of PFS in Group B and C patients classified according to the Simpson resection grade. For symbols, see Figure 1B.