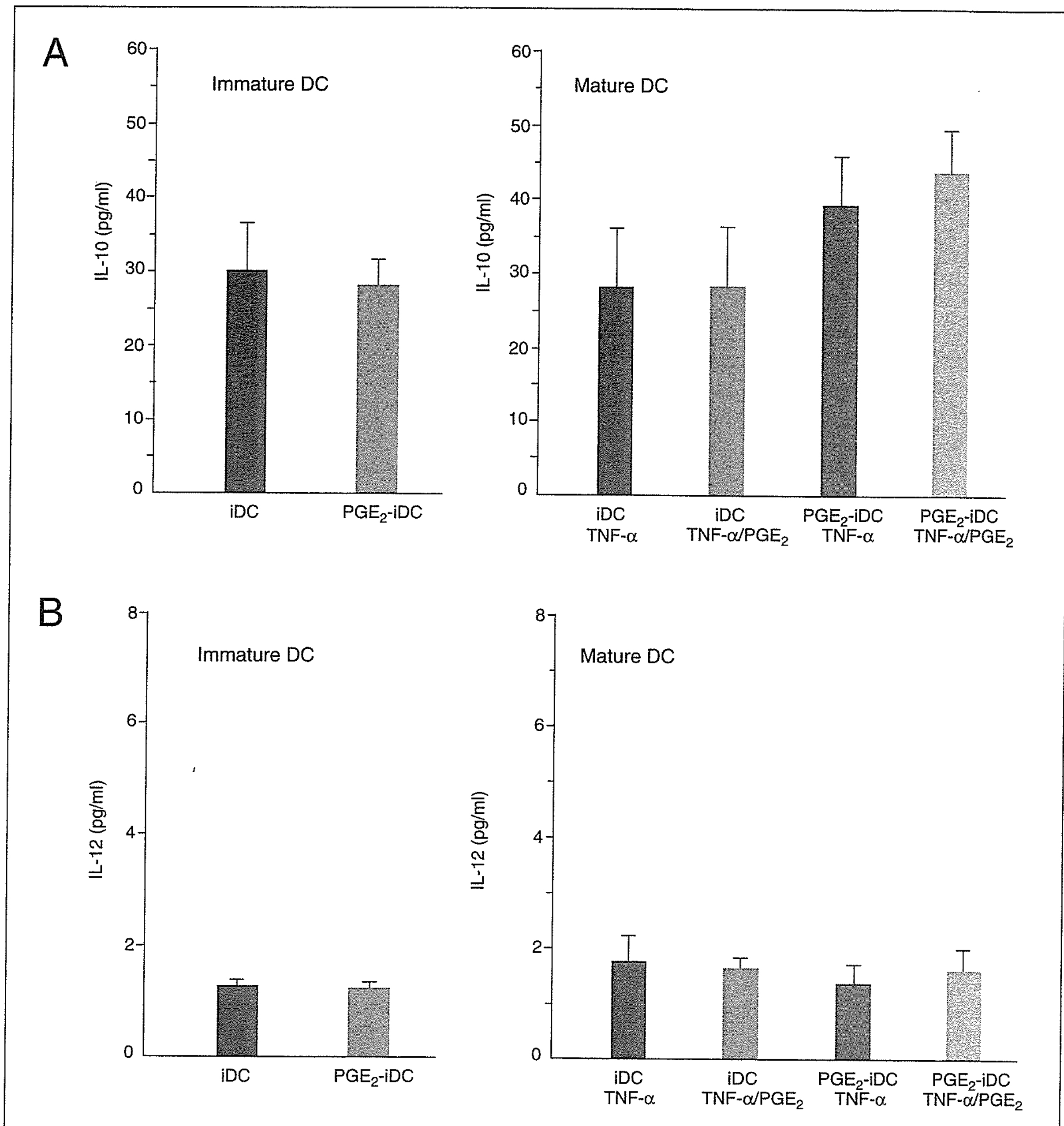


Figure 4. Measurement of IL-10 (A) and IL-12 (B) in supernatant of immature or mature DC culture without further stimulation. *DCs, regardless of the presence or absence of PGE_2, secreted very low levels of both cytokines in the basal state, suggesting that such secretion is not related to IFN-γ secretion. The results represent the mean (pg/ml) ± SE of at least seven independent experiments.*

potency of CTL itself was not augmented in the presence of PGE_2 (Fig. 5).

PGE_2 Did not Affect the Expression of Chemokine Receptors by Immature or Mature DCs

To evaluate whether the expression of chemokine receptors on DCs was affected by PGE_2, we performed a FACS analysis using mAbs to chemokine receptors in immature DCs, a transmigration test and calcium flux measurement using chemokines in mature DCs, and RT-PCR of CCR7 in mature DCs.

Phenotypic analysis of immature DCs using flow cytometry showed a high expression of CCR1 and CXCR4, with no difference between cell cultures with and without PGE_2, whereas CCR6 was not expressed at all in immature DCs (Fig. 6). In mature DCs, there was a high migration and a high calcium flux in response to MIP-3β, the ligand for CCR7, regardless of whether DCs were treated with

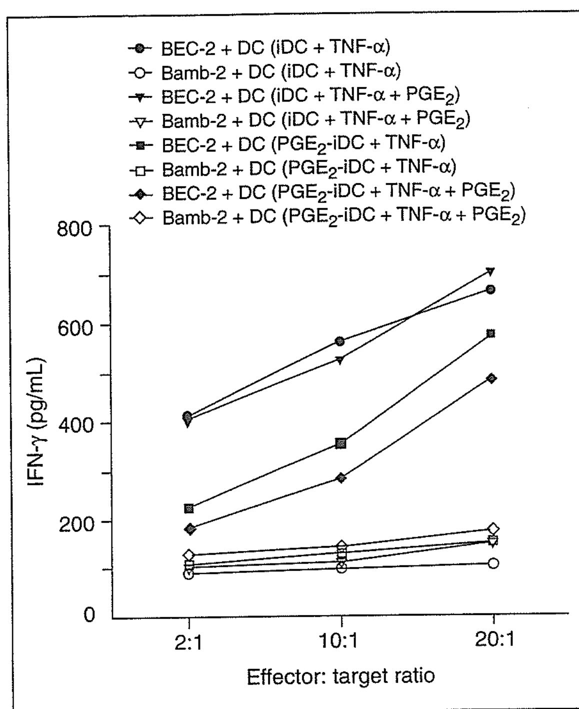


Figure 5. Autologous CD8⁺ T cells cocultured with mature DCs showed higher cytolytic activity against BEC-2 target cells at a high effector-to-target ratio than against Bamb-2 target cells. *However, the addition of PGE_2 did not affect the production of T cell IFN-γ by mature DCs. Autologous CD8⁺ T cells were cocultured with mature DCs pulsed with EBV-derived peptide, expanded with 100 U/ml IL-2, and, on day 8, restimulated with BEC-2 or Bamb-2 target cells at various effector-to-target ratios. After overnight culture, the IFN-γ concentration in the supernatant was measured by ELISA assay. The data are from one representative experiment.*

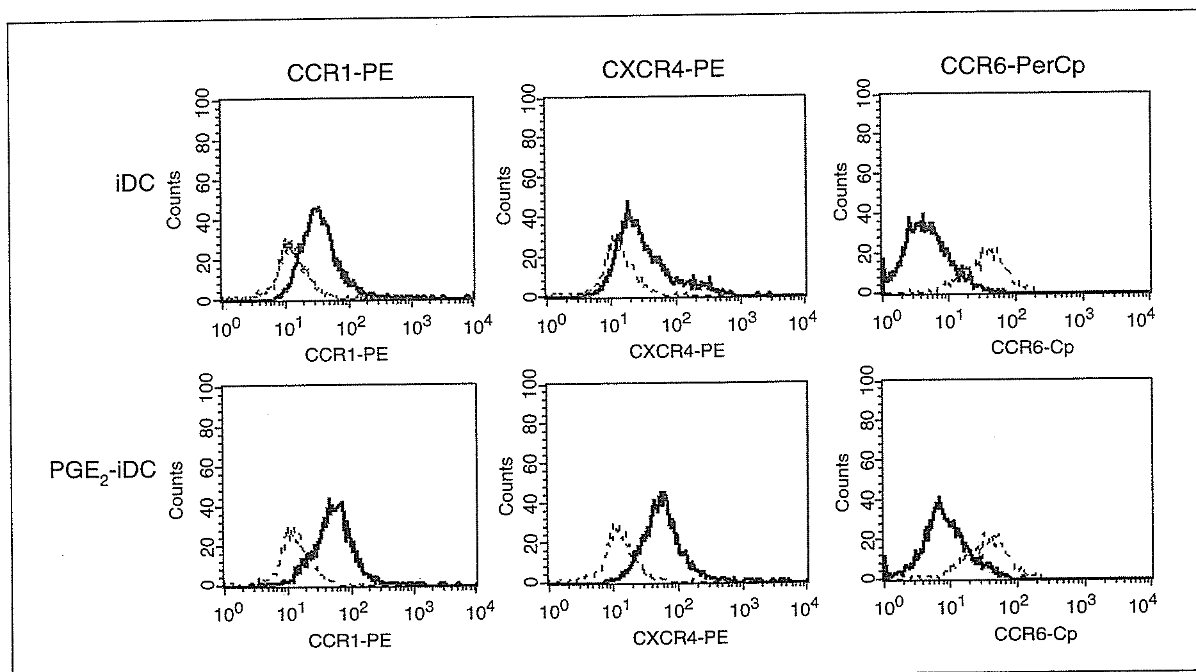
PGE_2 or not (Fig. 7). Additionally, RT-PCR to examine the expression of CCR7 showed similar positive reactions in mature DCs cultured with or without PGE_2 (Fig. 7).

Discussion

This study showed that, in the presence of PGE_2, DCs enhanced naïve T cells to polarize toward a Th1 response, independent of IL-12 secretion in the basal state, without any change in the expression of chemokine receptors on DCs. Moreover, PGE_2 induced a lower expression of CD1a and a persistently higher expression of CD14 in immature DCs, which is consistent with previous reports by other groups [14, 16, 17, 19]. Additionally, the observed phenotypic findings were similar to those for monocyte-derived macrophages cultured with M-CSF [27]. While monocyte-derived macrophages had little allostimulatory capacity, immature DCs derived from CD14⁺ cells with PGE_2 elicited a stronger MLR response than that of control cells cultured without PGE_2 [3, 28]. The reported allostimulatory capacity of T cells has varied when DCs were cultured in the presence of PGE_2 [15, 17, 18, 28].

This confusion is likely the result of different culture conditions, i.e., various cytokine combinations and culture

Figure 6. FACS analysis of chemokine receptor expression in immature DCs. *Immature DCs showed a high expression of CCR1 and CXCR4 (solid line), but no expression of CCR6 (solid line). However, the addition of PGE_2 did not affect the expression of these chemokine receptors on CD14⁺-cell-derived DCs. Dotted lines represent isotype-matched negative controls.*



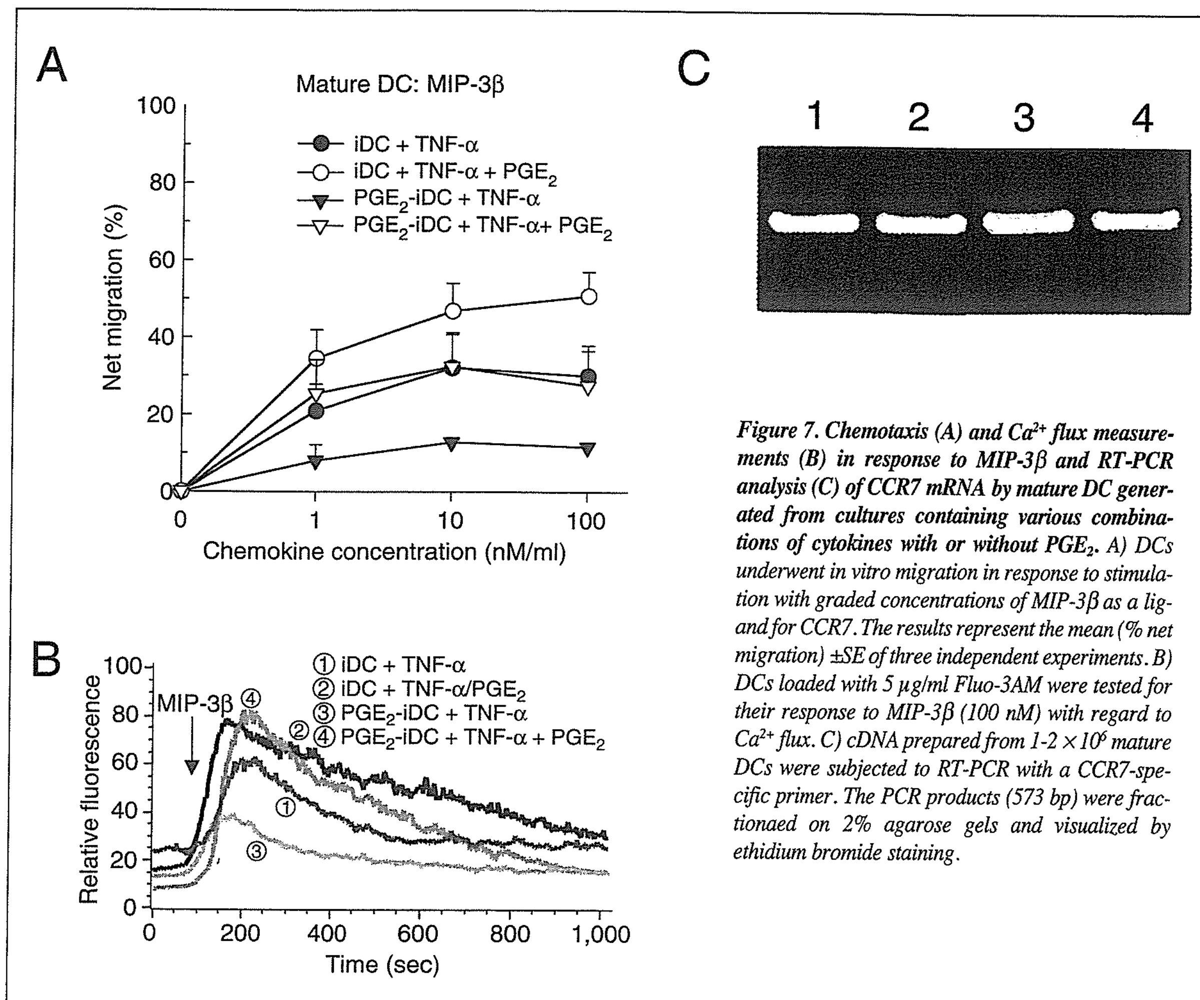


Figure 7. Chemotaxis (A) and Ca^{2+} flux measurements (B) in response to MIP-3β and RT-PCR analysis (C) of CCR7 mRNA by mature DC generated from cultures containing various combinations of cytokines with or without PGE_2. *A) DCs underwent in vitro migration in response to stimulation with graded concentrations of MIP-3β as a ligand for CCR7. The results represent the mean (% net migration) ±SE of three independent experiments. B) DCs loaded with 5 μg/ml Fluo-3AM were tested for their response to MIP-3β (100 nM) with regard to Ca^{2+} flux. C) cDNA prepared from 1-2 × 10^6 mature DCs were subjected to RT-PCR with a CCR7-specific primer. The PCR products (573 bp) were fractionaed on 2% agarose gels and visualized by ethidium bromide staining.*

media. Without the standardization of culture conditions, the results cannot be directly compared. In this study, immature DCs cultured in the presence of PGE_2 showed variable allostimulatory capacity that totally depended on the ratio of DC to T cells; a higher MLR response was seen at a higher ratio and a lower MLR response was seen at a lower ratio. In this study, DCs cultured with added PGE_2 during maturation had an enhanced allostimulatory capacity for $CD3^+$ T cells compared with controls without PGE_2.

Skewing of naïve T cells toward a Th1 or Th2 response is a crucial process in determining the ultimate outcome of the immune response, and this is affected by the culture environment of antigen-presenting DCs, as well as by the modulation of T-cell-receptor-mediated activation signals [29-31]. In this study, PGE_2 caused DCs to induce naïve T cells, which secreted IFN-γ independent of IL-12 secretion in the basal state, with a Th1 response. Hence, some of our findings are consistent with recently published reports that PGE_2 promoted a Th1 response [17, 19]. However, the results from these reports suggested that IL-12 production by DCs was crucial for increasing the production of IL-2 and IFN-γ [17-19]. This discrepancy regarding IL-12 production between our study and others might be explained by differences in the supernatant sample used for the measurement; we measured the supernatant of DCs cultured in the presence of PGE_2 without further stimulation, while others used supernatant after stimulating cells with various agents or supernatant of stimulated naïve T cells. A recent report supports our observation by finding only slight IL-12 secretion measured in samples in the basal state without further stimulation [32]. In addition, there is an interesting report that a high DC/T-cell ratio (1:4) resulted in a mixed Th1/Th2 response, while a low DC/T-cell ratio (1:300) induced T cells to become Th2 effectors, suggesting that the polarization of naïve T cells was influenced by their environment [33]. We showed that $CD14^+$-cell-derived DCs induced naïve T-cell differentiation with a Th1/Th2 response at a lower DC/T-cell ratio (1:300), although we used different DC maturation agents and a different stimulation of T cells by DCs than the previous report [33].

In contrast, *Kalinski* and coworkers [6, 14-16] reported that PGE_2 induced type 2-polarized DCs, which promote

the development of Th2 cells from naïve T cells via the inhibition of IL-12 production by DCs. Although *Steinbrink et al.* [19] argued that differences in the medium and cytokine combinations used for DC culture or a different ratio of T cells to DCs at stimulation produced contradictory results, we think that this discrepancy mainly resulted from the stimulating agents, such as cytokines or superantigen, used to produce DC maturation or to augment naïve T-cell stimulation, since we used a similar medium and ratio of T cells to DCs to those of *Kalinski* and coworkers. In that study, however, PGE_2 inhibited Th1 polarization of naïve T cells by DCs in the basal state compared with controls. Based on this observation, it is still possible that the induction of a Th2 response of naïve T cells by DCs is promoted by strong stimulating agents.

We evaluated the CTL response to examine the possible clinical application of our culture system, since DCs in the presence of PGE_2 induced a significant Th1 response. Unfortunately, the DCs cultured with PGE_2 did not produce a stronger CTL response than control cells cultured without PGE_2, thus excluding the possibility of clinical application. On the other hand, much attention has recently been focused on chemokine receptors as promising targets for clinical application. When immature DCs undergo maturation with various agents, CCR7, a constitutive chemokine receptor, is upregulated, and inflammatory chemokine receptors are downregulated, allowing the migration of DCs to lymphoid tissues [20-22]. In general, CCR6, a specific receptor for MIP-3β/liver and activation-regulated chemokine, is highly expressed in immature DCs derived from $CD34^+$ cord blood precursors, but not in $CD14^+$ peripheral blood monocyte-derived DCs [20-22]. Recently, *Yang et al.* [24] reported that CCR6 was also expressed in monocyte-derived DCs cultured in the presence of transforming growth factor-1 and that this contributed to the regulation of the trafficking of DCs, suggesting that DC culture conditions significantly affect the expression of chemokine receptors. In our study, immature DCs cultured in the presence of PGE_2 showed a high expression of CCR1 and CXCR4, but did not express CCR6 by a phenotype analysis. In addition, there was no difference in the expressions of CCR7 between mature DCs cultured with and without PGE_2, which supports the notion that PGE_2 does not directly influence the expression of chemokines.

In conclusion, our findings suggest that DCs cultured in the presence of PGE_2 enhance the differentiation of naïve T cells toward the Th1 type, independent of IL-12 secretion in the basal state, and that PGE_2 does not have any significant effect on chemokine receptor expression by DCs.

Acknowledgments

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Original Paper

Peroral Sustained-Release Indomethacin Treatment for Rectal Adenomas in Familial Adenomatous Polyposis: a Pilot Study

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ABSTRACT
Background/Aims: The efficacy of peroral sustained-release indomethacin on rectal adenomas in colectomized patients with familial adenomatous polyposis and its toxicity were evaluated preliminarily.
Methodology: A total of seven colectomized patients with familial adenomatous polyposis were treated with peroral sustained-release indomethacin at the usual clinical doses. The numbers of rectal polyps before and after treatment and approximately one year after cessation of treatment were counted using a flexible colonoscope and compared.
Results: After treatment, reductions of rectal polyp size were observed in all patients. Numbers of the rectal polyps also decreased significantly after treatment (median, 1; range, 0 to 30) compared with those before treatment (median, 19; range, 4 to 78) (P=0.023). However, Grade 4 anemia due to lower intestinal ulcers occurred in two patients. Moreover, one year after termination of the treatment, increase in the size and number of rectal polyps was observed in six patients.
Conclusions: Because of incomplete efficacy and severe toxicity, general use of this treatment for familial adenomatous polyposis patients must be deemed inappropriate.

KEY WORDS:
Familial adenomatous polyposis; Chemoprevention; Indomethacin

ABBREVIATIONS:
Familial Adenomatous Polyposis (FAP)

INTRODUCTION

In 1983, Waddell and Loughry first reported the efficacy of sulindac, a non-steroidal anti-inflammatory drug, for effecting regression of colorectal adenomas in familial adenomatous polyposis (FAP) patients (1). Similar results have been reported by the others for large series of cases (2-4) and several controlled trials (5,6). However, its effectiveness is incomplete and it is unlikely to replace colectomy as the primary therapy (5). In addition, sulindac causes severe adverse events in Japanese FAP patients (7), so that a more potent and less toxic agent must be sought.

Indomethacin, a related non-steroidal anti-inflammatory drug to sulindac, is also known to inhibit intestinal carcinogenesis in experimental animal models (8,9) and positive effects of indomethacin suppositories for treatment of rectal adenomas in FAP patients was recently suggested by two case series (10,11). Therefore, administration of indomethacin may be one treatment option.

The purpose of this pilot study was to evaluate preliminarily the efficacy of peroral sustained-release indomethacin on rectal adenomas in colectomized patients with FAP.

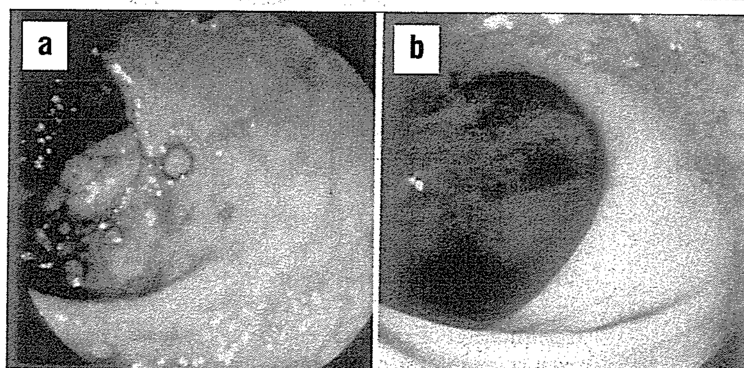

FIGURE 1 a) Sparse rectal polyposis before peroral sustained-release indomethacin treatment in a patient with familial adenomatous polyposis. The largest polyp was removed endoscopically at this time. **b)** After 4 months treatment, all of the remaining 24 rectal polyps have disappeared.

METHODOLOGY

Patients with FAP, who previously underwent total colectomy and ileorectal anastomosis, were enrolled in this study. Those with histories of peptic ulcers, liver dysfunction, a blood creatinine level of more than 1.5mg/dL, or thrombocytopenia were excluded. There were four men and three women with a median age of 36 (range, 23 to 44) years. A median interval between colectomy and enrollment in this study was 35 (range, 3 to 209) months. There were six patients with sparse polyposis **(Figure 1a)** and one with profuse polyposis **(Figure 2a)**. Informed consent was obtained from each patient.

The patients were given a daily dose of 75 to

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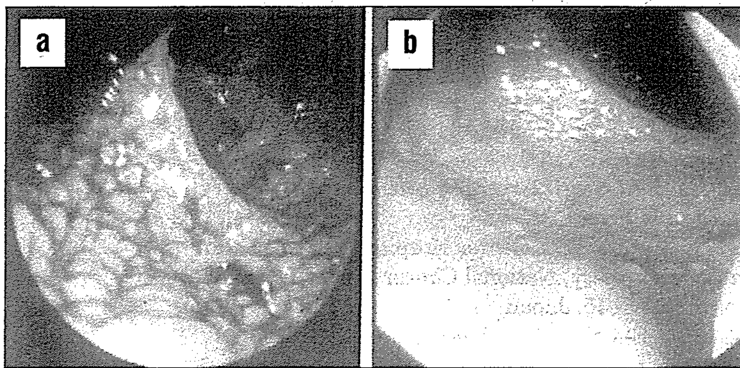

FIGURE 2 a) Profuse rectal polyposis before indomethacin treatment in another patient with familial adenomatous polyposis. **b)** After 7 months treatment, all polyps showed reduction in size and the numbers decreased from 78 to 30.

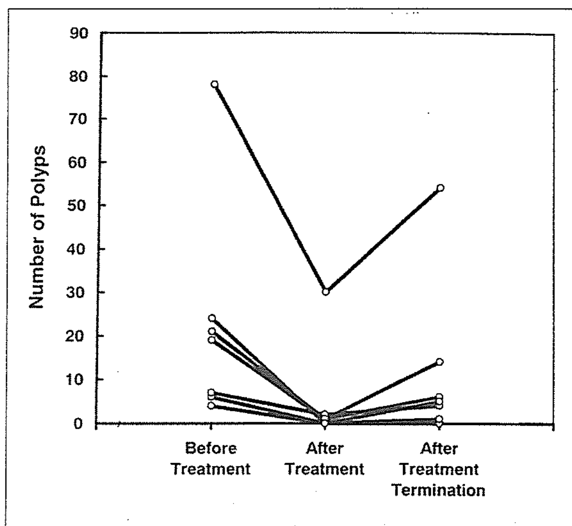

FIGURE 3 The number of rectal polyps in patients with familial adenomatous polyposis before and after peroral sustained-release indomethacin treatment and 1 year after its termination.

100mg of sustained-release indomethacin orally. The median dose per body surface area was 56 (range, 53 to 62) mg/m²/day. Treatment was continued until occurrence of grade 3 toxicity (12) or cancer, or the termination of the study. The patients were asked to report symptoms and signs of drug toxicity and underwent a physical examination every month. Complete blood counts, blood biochemistry, and endoscopy were performed when necessary. When abnormal symptoms, signs or values in the blood test attributable to indomethacin were noted, the treatment was discontinued.

The remaining rectum of the patients was examined with a flexible colonoscope before the initiation of indomethacin treatment and every three to five months thereafter. The numbers of rectal polyps before and after treatment and approximately one year after cessation of treatment were counted using a flexible colonoscope and compared. Any rectal lesion suspected of being cancer was biopsied.

Changes in the number of polyps were analyzed by *t*-tests for paired samples. All *P* values were two sided, and those less than 0.05 were considered to be statistically significant.

RESULTS

During the median period of 152 (range, 81 to 345) days, a median total indomethacin dose of 13.3 (range, 6.1 to 30.1) g was given to the patients. Compliance was excellent in all cases.

After treatment, reduction in size and number of rectal polyps was observed in all patients, including the six patients with sparse polyposis **(Figure 1b)** and the one with profuse polyposis **(Figure 2b)**. Overall, the numbers of rectal polyps decreased significantly after (median, 1; range, 0 to 30) as compared with before the treatment (median, 19; range, 4 to 78) (P=0.023) **(Figure 3)**, to 0 to 39% (median, 5%) of base-line values. In the patients with sparse polyposis, numbers of the rectal polyps decreased particularly markedly (median, 0.5; range, 0 to 2) as compared to before the treatment (median, 13; range, 4 to 27) (P=0.016) **(Figure 3)**. Rectal polyps in three patients with prior polyp numbers of 4, 6, and 24 **(Figure 1a)** disappeared completely **(Figure 3)**.

However, Grade 4 anemia (12) occurred in two female patients who took 54mg/m²/day indomethacin for 345 days and 56mg/m²/day for 81 days, respectively, and consequently, the treatment was discontinued. Gastroduodenal endoscopy revealed no lesions in the stomach and duodenum but proctoscopy demonstrated a linear ulcer on the ileorectal anastomosis in one. The anemia recovered under administration of a chalybeate. No adverse events were observed during 95 to 301 (median, 152) days of treatment in the other five patients. The pilot study was terminated because of this toxicity.

A median period of 373 (range, 222 to 451) days after termination of the treatment, rectal polyps were reevaluated. After treatment termination, increase in the size and number of rectal polyps was observed in six cases, the five with sparse polyposis and the one with profuse polyposis. In the other patient with sparse polyposis, rectal polyps did not reappear. The numbers of rectal polyps increased after treatment termination (median, 5; range, 0 to 54) compared with those after treatment **(Figure 3)**, although the difference was not statistically significant (P=0.07). No rectal cancers were found during the entire study period (median, 584 days; range, 294 to 764 days).

DISCUSSION

In this study, we chose oral sustained-release indomethacin as an agent for treatment of rectal adenomas in FAP patients for the following reasons. Firstly, although sulindac is known to be a potent drug in reducing colorectal adenomas in FAP patients (1-6), its effect is incomplete, and it is unlikely to replace colectomy as primary therapy (5). Moreover, the gastroduodenal adenomas, the precursors of gastroduodenal cancer which are causes of death in 6 to 8% of FAP patients (13,14), appear not to be affected by sulindac therapy (6). Therefore, a more potent agent is needed. Secondly, although there were few articles in the literature reporting toxicity of peroral

sulindac therapy in Western FAP patients (1-6), sulindac causes severe adverse events such as intestinal ulcers, perforating gastric ulcer, asymptomatic subcutaneous abscess, oligospermia in Japanese FAP patients (7). Consequently, a less toxic agent must be sought. Thirdly, there have been multiple reports of intestinal carcinogenesis inhibition by indomethacin in experimental animal models (8,9). Hirata *et al.* (10) and Hirota *et al.* (11) suggested that indomethacin suppository may be effective for reducing colorectal adenomas in FAP patients. Thus administration of indomethacin may be one treatment option. Fourthly, to treat gastroduodenal adenomas in addition to colorectal adenomas, peroral preparation may be preferable to suppository because of pharmacokinetics. Finally, sustained-release indomethacin is less toxic than ordinary indomethacin (15) and is only half the price of sulindac in Japan.

Although this is a small case series without controls, the results indicated that peroral sustained-release indomethacin, when used at the usual clinical doses effective for inflammation, brings about regression of many rectal adenomas in colectomized patients with FAP. After 3 to 11 months treatment, the numbers of polyps had decreased to 0 to 39% of base-line values and total disappearance was observed in three of the seven patients. These data may be comparable with the findings of Giardiello *et al.* who obtained a 44% decrease after nine months peroral sulindac treatment (5). However, as is observed with sulindac (2,3,5), re-appearance and regrowth of polyps were noted after termination of the treatment. Therefore, efficacy of this treatment is deemed incomplete.

In the majority of patients, no adverse events were observed during 3 to 10 months treatment and compliance with the treatment was excellent in all patients. However, the severe anemia observed in two cases means that long-term usage of this drug for chemoprevention is problematic. The cause of bleeding was not gastroduodenal ulcer, as commonly observed with indomethacin (16), but lower intestinal ulcer, a rare complication (17) also reported in a Japanese FAP patient treated with sulindac (7). This may be peculiar to Japanese FAP patients, because it has so far not been reported in any Western FAP patients (1-6).

We conclude that, although peroral sustained-release indomethacin may be an alternative to sulindac for selected patients, general use at the usual clinical doses for colorectal adenoma treatment and cancer chemoprevention in FAP is inappropriate. Nevertheless, further investigation on peroral indomethacin treatment at lower doses, which do not cause toxicity, is warranted.

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FOOTNOTE

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Pharmaceutical and Biomedical Differences between Micellar Doxorubicin (NK911) and Liposomal Doxorubicin (Doxil)

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The stability and biological behavior of an *in vitro* system of doxorubicin (DXR) entrapped in NK911, polymer micelles, was examined and compared with those of DXR entrapped in Doxil, polyethylene-glycol-conjugated liposomes. The fluorescence of DXR inside micelles or liposomes in an aqueous solution is known to be strongly quenched by the outer shells of the micellar or liposomal formation. Thus, by measuring the fluorescence intensity of DXR released from NK911 or Doxil, we could determine the stability of the micellar or liposomal DXR formation. Furthermore, NK911 was found to be less stable than Doxil in saline solution. In drug distribution experiments using an *in vitro* solid tumor model, when spheroids formed from two human colonic cancer lines, HT-29 and WiDr, and a human stomach cancer line, MKN28, were exposed to NK911, DXR was distributed throughout the spheroids, including their center. On the other hand, when the spheroids were exposed to Doxil, DXR was distributed only to the surface of the spheroids. It has been suggested that Doxil can deliver DXR to a solid tumor more efficiently than NK911 via the EPR (enhanced permeability and retention) effect, because Doxil may be more stable in plasma than NK911. On the other hand, DXR packed in NK911 may be distributed by diffusion to cancer cells distant from the tumor vessel, because NK911 can leak out of the tumor vessel and may be able to release free DXR more easily than Doxil. It has been suggested that drug carrier systems such as liposomes and micelles should be selected appropriately bearing in mind the characteristics of the tumor vasculature and the tumor interstitium.

Key words: NK911 — Drug delivery system — Polymer micelles — Doxil — Doxorubicin

It has been determined from pathological, pharmacological and biochemical studies, that in general, solid tumors possess the following pathophysiological characteristics: hypervascularity, irregular vascular architecture, potential for secretion of vascular permeability factors, and absence of effective lymphatic drainage that prevents efficient clearance of macromolecules accumulated in the solid tumor tissues.[1–6] It has been suggested that these characteristics, which are unique to solid tumors, constitute the basis of the enhanced permeability and retention (EPR) effect.[1,2] Moreover, macromolecules as well as small particles have relatively prolonged plasma half-lives because they are too large to pass through normal vessel walls unless they are trapped by the reticuloendothelial system (RES). However, they can extravasate into and accumulate within tumor tissues through the EPR effect. On the other hand, conventional low-molecular-weight anticancer agents are usually eliminated before they reach the tumor tissue to exert their cytotoxic effects.[1,2]

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To use the EPR effect to advantage, several techniques have been developed to modify the structure of drugs and to construct carriers. Liposomes with a polyethylene glycol (PEG) coating have proved to be very successful as a drug carrier system. PEG is electrically neutral and is not recognized by the RES in the liver or spleen; this forms the basis of the so-called "stealth effect."[7–9] Liposomal drugs exhibit reduced clearance and prolonged plasma half-lives due to the stealth effect.[10] Doxil is long-circulating pegylated liposomes containing doxorubicin (DXR), and has received the Food and Drug Administration (FDA)'s approval for use in the treatment of Kaposi's sarcoma[11,12] or ovarian cancer,[13] because its clinical benefits were clearly shown in recent clinical trials, including a phase-III randomized trial. Biodistribution studies using in DTPA (diethylenetriaminepentaacetic acid)-labeled pegylated liposomes have demonstrated selective tumor accumulation of the constituent drug in patients with advanced head and neck, lung or breast cancer.[14]

Polymeric micelles have also been utilized as a drug carrier system. Initially, Yokoyama *et al.* succeeded in constructing a micelle-forming polymeric drug, PEG-

poly(aspartic acid) block copolymer, conjugated with DXR.[15] PEG constituted the outer shell of the micelle, conferring the stealth property on the drug preparation. The poly(aspartic acid) chain is a hydrophobic chain and the poly(aspartic acid)-DXR conjugates form the hydrophobic core of the micelles in aqueous media. This original form of micellar DXR contained two entrapped components, DXR monomers and DXR dimers, in the inner core. It has been reported that it is the DXR monomers, and not DXR dimers, which play a major role in the antitumor activity of this drug preparation. DXR dimers, on the other hand, were considered to contribute to stabilization of the micellar DXR conformation.[16] However, it has been observed that freeze-dried samples of this micellar DXR become insoluble after prolonged storage, because of the presence of DXR dimers in this formulation.[17] To improve the solubility and stability of micellar DXR, therefore, a new type of polymeric micellar preparation, namely, NK911, containing DXR monomers alone, was generated.[17] NK911 has a small particle size, with a diameter of approximately 40 nm. After carrying out pre-clinical studies, we started a phase I clinical trial of NK911 to determine its usefulness in the treatment of various kinds of solid tumors.

Both liposomal and micellar DXR were found to have longer plasma half-life, to accumulate more efficiently in tumors due to the EPR effect and to show stronger antitumor activity in comparison with free DXR, in studies conducted in mice.[15–17] Previous studies, however, have shown that the AUC of NK911 in both plasma and the tumor is lower than that of Doxil. Therefore, in the present study, we examined the pharmaceutical and biomedical differences between NK911 and Doxil and explored the possible clinical advantages of NK911.

MATERIALS AND METHODS

Drugs The DXR-incorporating polymer micelles NK911 were procured from Nippon Kayaku Co., Ltd. (Tokyo). The structure and physical characteristics of this formulation have been described previously.[17] In brief, the micelle carrier NK911 consists of the block copolymer of PEG (MW 5000) and polyaspartic acid (about 30 units). The overall net charge of NK911 is neutral on the surface of the polymeric micellar vehicles due to the presence of PEG in the outer layer. The particle size (mean diameter 41.9 nm) and the narrow size distribution of NK911 remained unchanged after freeze-drying. The DXR-incorporating liposomes, Doxil, were purchased from ALZA Corp. (Mountain View, CA), and DXR hydrochloride ($C_{27}H_{29}NO_{11}$•HCl) was purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals used were of reagent grade and were used as purchased.

Cell lines and multicellular aggregates (spheroids) The human colon cancer cell lines HT-29 and WiDr, and the human stomach cancer line MKN-28, were cultured in D-MEM (Dulbecco's modified Eagle's medium)/10% FBS (fetal bovine serum) containing 15 mg/liter gentamycin sulfate and 50 mg/liter ampicillin sodium, at 37°C in a humidified atmosphere with 5% CO_2. Each of the cell lines was seeded onto 0.5% agarose-coated 10-cm dishes containing the medium described above, and incubated under similar conditions. One week later, several sizes of spheroids were noted to have grown in the dishes. For the drug distribution experiments, 200- to 500-μm sized spheroids were used.

Stability of NK911 and Doxil in aqueous solution Free DXR, NK911 and Doxil were incubated in saline in disposable plastic cuvettes at a DXR dose-equivalent of 20, 2.0 and 0.2 μM, for 0, 0.5, 1, 3, 6, 24, 48 and 72 h at 37°C. After each incubation time, the fluorescence intensity of each solution was measured using a fluorescence spectrophotometer (F-2500, Hitachi, Tokyo) at an excitation wavelength of 480 nm and emission wavelength of 550 nm. Since the fluorescence of DXR inside micelles or liposomes is known to be strongly quenched by the outer shells of the micellar or liposomal formation, it is possible to determine the stability of the micellar or liposomal DXR formulation by measuring the fluorescence intensity of DXR released from it.

Distribution studies of free DXR, NK911, and Doxil in the spheroids Spheroids derived from HT-29, WiDr and MKN-28 were exposed to free DXR, NK911 and Doxil, at 37°C at a DXR dose-equivalent of 20, 2.0 or 0.2 μM, for 1 h or 24 h. After the drug exposure, the spheroids were washed twice with phosphate-buffered saline (PBS(−)) and divided into 3 groups. Spheroids incubated without any drug for each aforementioned incubation time were used as controls. The first group was embedded in O.C.T. Compound (TISSUE-TEK, Miles, Inc., Elkhart, IN) and frozen at −80°C until use. The frozen sections were examined under a fluorescence microscope (BX50, DP50, Olympus, Tokyo) at an excitation wavelength of 470 nm and emission wavelength of 560 nm, to evaluate the distribution of DXR in the spheroids.

The second group was mixed with 1 ml of 0.1 M ammonium chloride buffer (pH 9.0) and homogenized. Then, 1 ml of the homogenized sample was transferred to a silicone-coated glass tube (the remainder of the homogenized solution was used for protein assay), and 50 μl of 2 μg/ml daunorubicin was added as an internal standard. Then, 5 ml of CH_3Cl_3/CH_3OH (2/1,v/v) was added to the mixed solution, which was shaken vigorously in a vortex mixer for 1 min and centrifuged at 10 000 rpm, to separate the buffer from the organic material. The organic layer was collected and transferred to another tube for evaporation under nitrogen gas flow at 40°C in a water bath. After evaporation of the sample, the precipitate was dissolved in 100 μl of DMF (N,N-dimethylformamide). Then, the solu-

tion was filtered through "Ultrafree"-MC, a low-binding hydrophilic 0.4-μm PTFE membrane (Millipore Corp., Bedford, MA) and subjected to reverse-phase HPLC to detect DXR.

As samples for the standard curve, 0.5, 1.0, 5.0, 10, 50, 100, 500, 1000 μg of DXR was added to drug-free homogenized spheroid solution, and treated as described previously before being subjected to HPLC to obtain a standard curve. The reverse-phase HPLC was carried out using a Gulliver 1500 series HPLC system (JASCO Corp., Tokyo) equipped with a PU-1580 Intelligent Pump, at a flow rate of 1.0 ml/min at 40°C using a CAPCELL PAK C18 SG300 column, 4.6 mm ID×150 mm, 5 μm, Shiseido Fine Chemicals (Tokyo). The fluorescence intensity of DXR in the samples was detected using an FP-1520 (JASCO Corp.).

Two types of gradient conditions between solution A (25 m*M* sodium phosphate buffer, pH 4.0) and solution B (CH_3CN) were used. For free DXR samples and standard curve samples, solution B was graded from 22% to 30% from 0 to 10 min, 30% of solution B and 70% of solution A were maintained from 10 to 14 min, solution B was graded from 30% to 22% from 14 to 15 min, and finally, 22% of solution B and 78% of solution A were maintained from 15 to 23 min. For the samples of Doxil and NK911, solution B was graded from 22% to 30% from 0 to 10 min, 30% of solution B and 70% of solution A were maintained from 10 to 14 min, solution B was graded from 30% to 80% from 14 to 17 min, 80% of solution B and 20% of solution A were maintained from 17 to 22 min, solution B was graded from 80% to 22% from 22 to 23 min, and finally, 22% of solution B and 78% of solution A were maintained from 23 to 35 min. In the latter gradient conditions for liposomes and micelles, solution B was graded up to 80% in order to wash out the polymers from the column. Ten microliters of the homogenates (the remaining samples were used for HPLC) were put into 96-well plates with 100 μl of filtered dye, diluted Dye Reagent Concentrate (Bio-Rad Protein Assay Dye Reagent Concentrate with 4 volumes of deionized water); 500, 400, 300, 200, 100 and 50 μl/ml of bovine albumin were used for obtaining the standard curve. After a few minutes, the optical density was measured using a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) at a wavelength of 595 nm. The amount of DXR distributed in the spheroids was expressed as μg DXR per mg cell protein.

Colony formation assay for cells from spheroids treated with each drug The third group of spheroids was treated with 0.5 ml of 0.25% trypsin-0.02% EDTA/PBS(−) solution in 1.5 ml Eppendorf tubes. After incubation for a few minutes, single-cell suspensions were obtained by tapping the tube. After addition of 0.5 ml of medium, the cells in the cell suspensions were counted using a Coulter counter (Z1, Coulter Corp., Miami, FL), and adjusted to 60 000 cells/ml by adding medium. One part of the 60 000 cells/ml cell suspension was mixed with 2 parts of medium containing 0.5% agarose to prepare 0.33% agarose gel containing 20 000 cells per ml. Then, 0.5 ml of the cell solution, containing 10 000 cells, was plated in triplicate as the upper layer in six-well plates coated with 0.55% agarose gel in the medium as the lower layer. The plates were incubated under the same incubation condi-

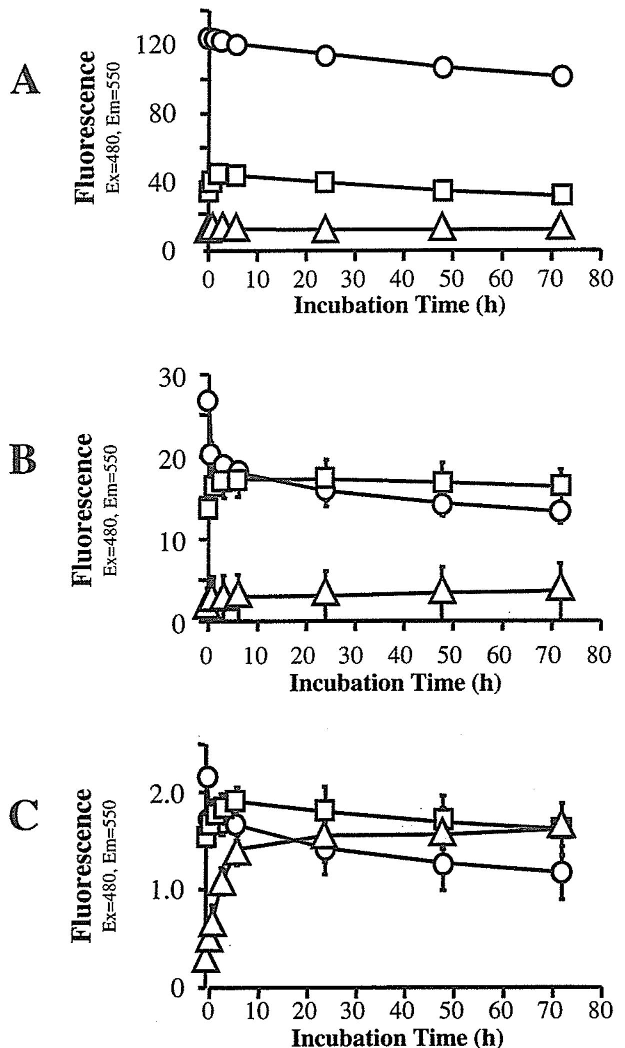

Fig. 1. Change in the fluorescence intensity of free DXR, NK911 and Doxil in PBS solution at 37°C. Free DXR (○), NK911 (□) and Doxil (△) were incubated in PBS at 37°C at a DXR dose-equivalent of 20 μM (A), 2 μM (B) and 0.2 μM (C). The fluorescence intensity of each solution was measured using a fluorescence spectrophotometer, at an excitation wavelength of 480 nm and emission wavelength of 550 nm, after 0.5, 1, 3, 6, 24, 48, and 72 h of incubation.

tions as mentioned above. One week later, colonies larger than 20–30 μm were counted in three microscopic fields. The colony formation ratio was calculated as the colony number in the test samples divided by that in the control samples×100 (%).

Statistical methods Data were compared using Fisher's PLSD test. *P* values of 0.05 or less were considered to denote statistical significance.

RESULTS

Stability of NK911 and Doxil in aqueous solution Neither NK911 nor Doxil at the DXR dose-equivalent of 20 *μM* released free DXR until at least 72 h in saline. At a DXR dose-equivalent of 2 *μM*, NK911 released almost all of the packed DXR by 3 h, while Doxil released only a very small amount of DXR. At the DXR dose-equivalent of 0.2 *μM*, both NK911 and Doxil released all of the DXR from each capsule by 3 h (Fig. 1).

Distribution studies of free DXR, NK911, and Doxil in the spheroids In the distribution experiments using spheroids made from HT29 cells, when the spheroids were exposed to free DXR, NK911 or Doxil for 1 h, red fluorescence originating from DXR was not clearly observed in any drug case (Fig. 2A). When the spheroids were exposed to free DXR or NK911 for 24 h, the DXR was clearly distributed throughout the spheroids, including their center. On the other hand, when the spheroids were incubated with Doxil for 24 h, weak fluorescence of DXR was observed only on the surface of the spheroids (Fig. 2B). Similar results were obtained when spheroids derived from WiDr or MKN-28 were treated with each of the drug preparations (data not shown).

These microscopic observations were confirmed quantitatively by measuring the amount of DXR extracted by reverse-phase HPLC from each of the spheroid groups exposed to free DXR, NK911 and Doxil (Fig. 3). When the spheroids were exposed to free DXR, NK911 or Doxil for 1 h at a DXR dose-equivalent of 20 *μM*, the total DXR contents in the spheroids were determined to be 0.851±0.411 μg/mg protein, 0.413±0.123 μg/mg protein or 0.009±0.002 μg/mg protein, respectively. The DXR content in the spheroids following exposure to NK911 was 46-fold higher than that following exposure to Doxil (P=0.0242). In the case of exposure of the spheroids to free DXR, the DXR content in the spheroids was 2-fold higher and 95-fold higher than that following exposure to NK911 (P=0.0162) and Doxil (P=0.0002), respectively. The DXR contents were 0.045±0.007 μg/mg protein, 0.051±0.031 μg/mg protein and 0.002±0.001 μg/mg protein following exposure of the spheroids to free DXR, NK911 and Doxil for 1 h at the DXR dose-equivalent of 2 *μM*, respectively. Thus, in terms of the DXR content in the spheroids, there was no significant difference between the

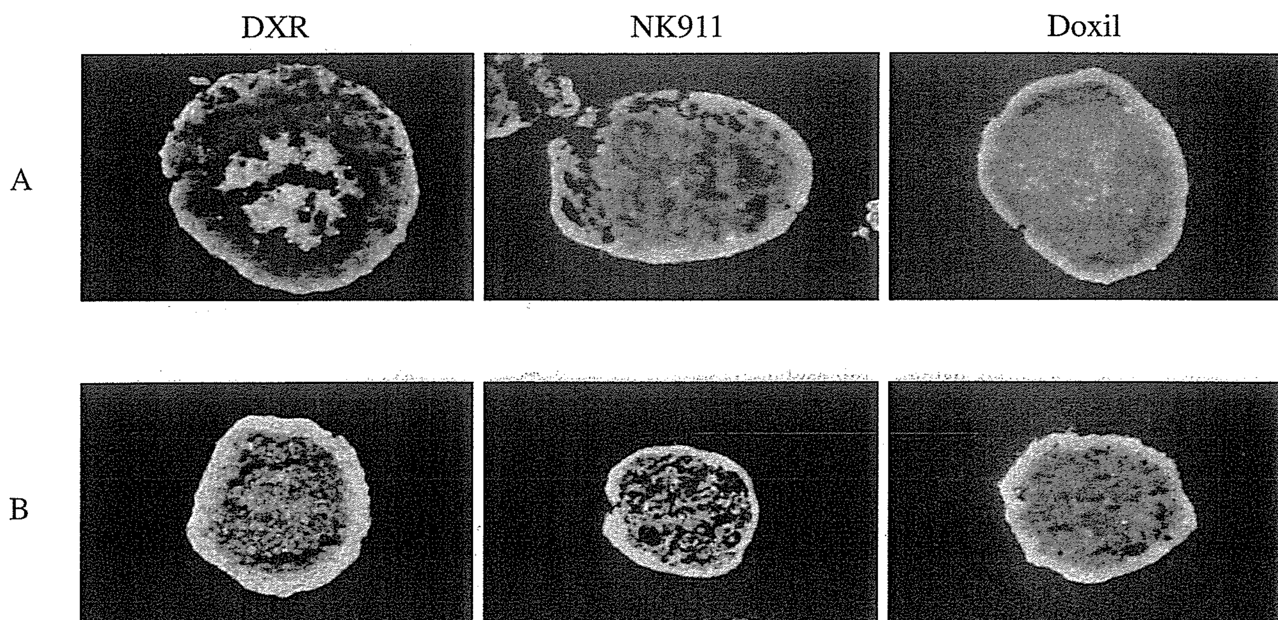

Fig. 2. DXR distribution in spheroids derived from HT-29 cells after exposure to free DXR, NK911 and Doxil. The spheroids were exposed to free DXR, NK911 or Doxil at a DXR dose-equivalent of 20 *μM* for 1 h (A) and 24 h (B) at 37°C. The frozen sections were examined using under a fluorescence microscope at the excitation wavelength of 470 nm and emission wavelength of 560 nm.

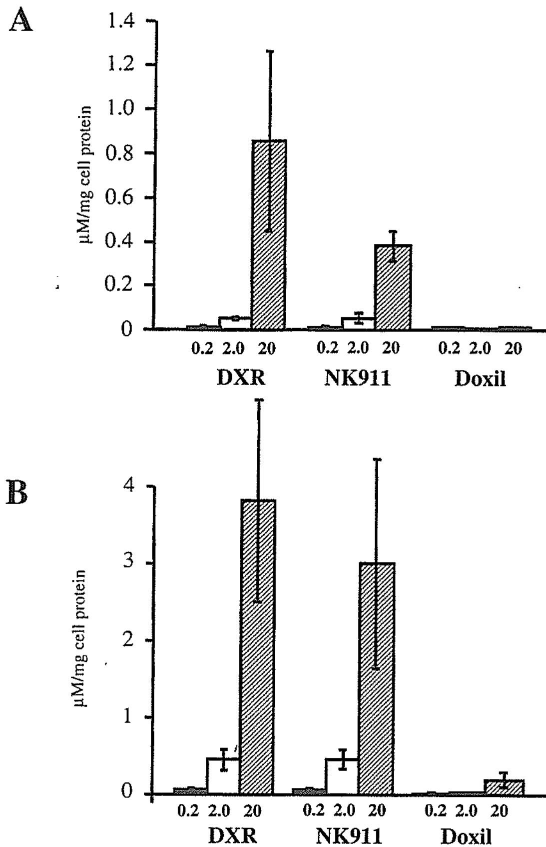

Fig. 3. Quantification of DXR distributed in spheroids derived from HT-29 after their exposure to free DXR, NK911 or Doxil. After their exposure to free DXR, NK911 or Doxil at the DXR dose-equivalent of 20 *μM* (▨), 2 *μM* (□) and 0.2 *μM* (■) for 1 h (A) or 24 h (B), the spheroids were washed and homogenized. The homogenized solutions were subjected to the DXR extraction procedure and the extracts were subjected to reverse-phase HPLC to quantify the DXR content in the spheroids.

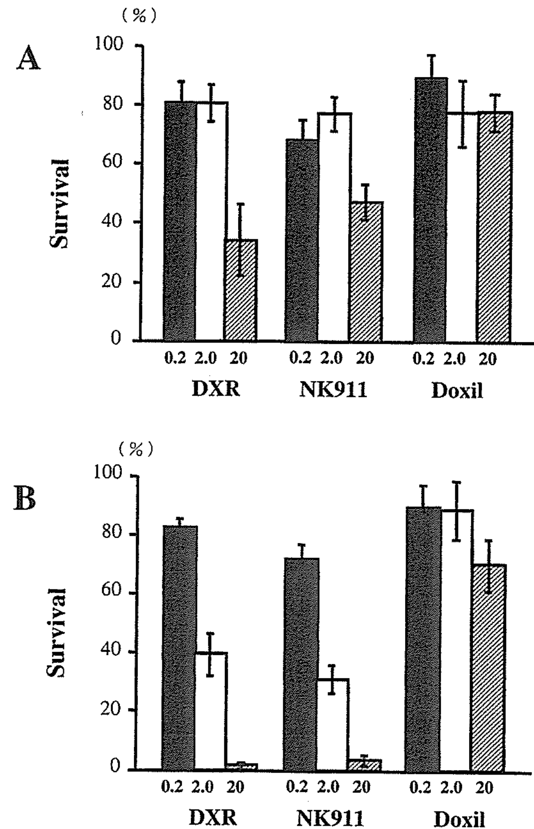

Fig. 4. Colony formation assay for cells derived from HT-29 spheroids after their exposure to free DXR, NK911 or Doxil. After exposure to free DXR, NK911 or Doxil at a DXR dose-equivalent of 20 *μM* (▨), 2 *μM* (□) or 0.2 *μM* (■) for 1 h (A) or 24 h (B) the spheroids were washed and trypsinized to prepare single-cell suspensions. The cell suspensions were plated in triplicate onto soft agar. A week later, the colonies in one microscopic field were counted.

NK911 and free DXR treatment groups. However, the DXR content in the spheroids in the case of the Doxil treatment group was 23-fold and 26-fold lower than those in the free DXR and NK911 treatment groups, respectively (P=0.0057 for Doxil vs. DXR and P=0.0053 for Doxil vs. NK911). The DXR contents were 0.009±0.005 *μ*g/mg protein and 0.010±0.002 *μ*g/mg protein when the spheroids were exposed to free DXR and NK911 spheroids for 1 h at a DXR dose-equivalent of 0.2 *μM*, respectively. Almost no DXR was detected in the spheroids exposed to Doxil at this DXR dose-equivalent (Fig. 3A).

When the spheroids were treated with free DXR, NK911 and Doxil for 24 h at DXR dose-equivalents of 20 *μM*, 2 *μM*, 0.2 *μM*, similar results to the case of 1-h treatment were obtained. Namely, the DXR distribution was significantly higher in the case of free DXR or NK911 treatment than that in the case of Doxil treatment, but there was no significant difference between the free DXR and NK911 treatment groups at any dose of DXR (Fig. 3B). The major difference between the 1-h treatment and 24-h treatment groups was that the DXR content in the spheroids was higher following 1-h exposure to free DXR at 20 *μM* dose as compared to that following 1-h exposure to NK911 at a DXR dose-equivalent dose of 20 *μM*, but there was no significant difference between the two following exposure to a DXR dose-equivalent of 20 *μM* for 24 h.

Colony formation assay for cells from the spheroids treated with each drug Colony formation assay for cells from the spheroids of HT-29 was conducted to determine the cytotoxicity of free DXR, NK911 or Doxil against *in vitro* solid tumor model spheroids. As shown in Fig. 4, on

the whole, a higher cytotoxic effect was obtained in the group treated with free DXR or NK911 as compared with that treated with Doxil. The results were roughly consistent with those from the drug distribution experiments. When the spheroids were treated with each drug for 1 h, there was no significant difference among the 3 drug preparations for a DXR dose-equivalent of 0.2 μM or 2 μM. However, following 1-h exposure to a DXR dose-equivalent of 20 μM, free DXR showed the strongest cytotoxic effect, followed by NK911. In the case of 24-h exposure, while free DXR and NK911 were superior to Doxil, there was no significant difference between free DXR and NK911 treatment at any dose of DXR.

DISCUSSION

Several DDS drugs have been approved by the regulatory authorities for the treatment of cancer, including poly(styrene-maleic anhydride)-neocarzinostatin (SMANCS) in Lipiodol for the treatment of hepatoma[18] and PEG-L-asparaginase for the treatment of acute lymphocytic leukemia.[19] Regarding liposomal anthracycline formulations, extensive clinical trials of two liposomal preparations of DXR, Doxil and TLC-99,[20] and one preparation of daunorubicin, DaunoXome[21] have been conducted. Several studies have reported that liposomal preparations of anthracyclines are associated with attenuated toxicity, including reduced cardiac toxicity, while the efficacy of the parent anthracyclines is retained, or even enhanced.[20–22] Among such preparations, Doxil has already been approved for use in the treatment of Kaposi's sarcoma and ovarian cancers by the FDA in the USA. To expand the uses of liposomal preparations of anthracyclines, several clinical trials to determine their usefulness in the treatment of other cancers are currently in progress.

Ringsdorf *et al.* proposed that AB block copolymer-drug conjugates might form micellar structures that could improve the drug solubility.[23] Then, the utility of polymeric micelles in cancer chemotherapy was demonstrated for the first time with DXR-incorporating polymeric micelles in the early 1990's.[15] Since the size of these micellar structures was large enough to evade renal excretion and because of the covering of the outer shell of the micelle with PEG, preventing nonspecific capture by the RES, DXR-incorporating polymeric micelles have a long plasma half-life, which permits large amounts of DXR-incorporating micelles to reach the target sites and exert their EPR effect. These original DXR-incorporating polymeric micelles decreased the toxicity of DXR significantly in terms of body weight change and blood biochemical characteristics, and yet exhibited superior *in vivo* antitumor activity against several solid tumors in comparison with free DXR in experiments conducted in mice.[15] However, it was found that freeze-dried samples of the original micellar DXR became water-insoluble after prolonged storage, because of the existence of the DXR dimers in the preparation. To overcome this problem, a new type of polymeric micelles was generated, containing only DXR monomer, and was found to dissolve in water easily even after prolonged storage in the freeze-dried condition. This new type of DXR-incorporating micelles, named NK911, had a shorter plasma half-life than the original micellar DXR, because NK911 was not stable in the bloodstream due to the lack of DXR dimers. Nakanishi *et al.* reported that the area under the concentration curve (AUC) of the DXR incorporated in NK911 in the plasma was 28.9-fold higher than that of free DXR, in experiments conducted using C-26-colon-carcinoma-bearing mice.[17] The AUC of the DXR in NK911 in the tumor of mice inoculated subcutaneously with C-26 cells was 3.4-fold higher than that of free DXR.[17] While the AUC in plasma of the DXR incorporated in Doxil was 237-fold higher than that of free DXR,[24] Vaage *et al.* reported that the relative values of the AUC in the tumor for free DXR and Doxil were 36.5 and 919, respectively, from experiments conducted using human prostate carcinoma (PC-3)-bearing nude mice. This represents a 25-fold increase in the concentration of the drug at the tumor site.[25] Although each study was conducted independently using different tumors and rodent models, the findings suggest that Doxil has a longer plasma half-life and higher AUC in the plasma than NK911; therefore, Doxil can accumulate in solid tumor tissue more efficiently than NK911, based on the EPR effect.

In the current study, at higher concentrations, such as a DXR dose-equivalent of 20 μM, neither NK911 nor Doxil released free DXR until at least 72 h in PBS solution. At a DXR dose-equivalent of 2 μM, NK911 released almost all the packed DXR within it by 3 h, while Doxil released only a small amount of DXR. These findings imply that NK911 is less stable than Doxil in aqueous solution. In addition, the present findings suggest that Doxil can deliver DXR to a solid tumor via the EPR effect better than NK911, probably because it is more stable in plasma than NK911.

Jain *et al.* extensively studied factors interfering with drug delivery to solid tumors. They reported that the convective passage of large drug molecules into the core of solid tumors could be impeded by abnormally high interstitial pressures in solid tumors. Therefore, small-molecular-weight anticancer agents (with a molecular weight lower than 2000 daltons) are superior for the treatment of solid tumors, because they can leave the tumor blood vessels and migrate into the core of the tumor by diffusion. However, low-molecular-weight anticancer agents can also be harmful to normal cells because they can leak out of normal blood vessels. Therefore, Jain suggested that one useful strategy for evading the barriers to drug dispersion

would be to inject patients with drug carriers, such as liposomes, filled with low-molecular-weight drugs. In this system, the liposomes should have time to exit from the leaky areas of blood vessels and reach reasonably high levels in the surrounding interstitium. The important thing in Jain's concept of a liposomal carrier system is that the liposomes should release the low-molecular-weight drugs packed within them gradually, so that the drug can be dispersed throughout the tumor.[26] Some interesting findings were reported by Unezaki *et al.*,[28] who succeeded in visualizing the extravasation of PEG-liposomes into a solid tumor using fluorescence-labeling. In a study using C-1300-bearing mice, they showed that while the fluorescence-labeled PEG-liposomes localized immediately around the tumor vessel wall after extravasation, they could not be detected in normal tissue. Thus, the PEG-liposomal system possesses the ability to deliver DXR more efficiently, but to obtain satisfactory antitumor effect, liposomes have to release free DXR at the site of extravasation.

In our current distribution study of NK911 using spheroids, when spheroids were exposed to free DXR or NK911, DXR was distributed throughout the spheroids, including their core. On the other hand, when the spheroids were exposed to Doxil, DXR was distributed only to the surface of the spheroids. These findings indicate that NK911, but not Doxil, can easily release DXR, which is then distributed throughout the spheroids by diffusion, because NK911 is not as stable as Doxil in the medium. These results were demonstrated quantitatively using HPLC, and visually by fluorescence microscopy for the spheroids in the experiment. In addition, it was confirmed by colony formation assay that NK911 exerts stronger cytotoxic effects than Doxil against spheroid models, because NK911 releases much more free DXR, and the DXR is distributed throughout the spheroids, as compared with the findings observed in the case of Doxil. Moreover, when the spheroids were exposed to each drug at a DXR dose-equivalent of 20 μM for 1 h, the best result was seen with free DXR treatment, followed by that with NK911 treatment, and the least significant result was noted with Doxil treatment, in terms of both DXR content in the spheroids and colony formation. When the spheroids were exposed to each drug at a DXR dose-equivalent of 20 μM for 24 h, the lowest DXR content in the spheroids and the lowest antitumor effect were obtained following Doxil treatment, and there was no significant difference between the results following free DXR and NK911 treatment. These results indicate that the micellar formation of NK911 was preserved during the first hours of exposure, although NK911 released DXR more gradually. However, after 24 h, the micellar formation of NK911 decayed, and the amount of DXR released was equivalent to that following free DXR exposure. Taking all these findings together, it is suggested that Doxil can deliver DXR to a solid tumor more efficiently than NK911 via the EPR effect, because it is more stable in the bloodstream and has a higher AUC in the plasma than NK911. However, DXR from NK911 is distributed more efficiently to cancer cells distant from the tumor vessel than DXR from Doxil once NK911 extravasates from the tumor vessel. Although there is no concrete evidence regarding how significant these differences between NK911 and Doxil might be clinically, NK911 may be more effective against cancers having a rough tumor vessel network, because of the presence of an abundant collagen-rich matrix. Such cancers include scirrhous stomach cancer, pancreatic cancer, inflammatory breast cancers, the so-called intractable cancers. Unfortunately, no experimental tumor models have been established as yet which can represent collagen-rich and intractable human tumors. Therefore, there is no way of determining the clinical significance of NK911 or Doxil other than conducting a clinical trial.

There are some advantages of the polymeric micellar system over the liposomal system. In the liposomal system, strongly hydrophobic drugs are preferentially retained in the lipid bilayer of liposomes, not in the inner aqueous area. The hydrophobic drugs incorporated in the lipid bilayer may destabilize the liposomal structure and so incorporation must be limited to a small amount of drug per liposome. In contrast to the liposomal system, the polymeric micelle system can incorporate hydrophobic drugs by utilizing hydrophobic interactions between the hydrophobic drug and the inner core, which is composed of the hydrophobic chain of block copolymers. In addition to DXR, cisplatin,[29] taxol[30] or KRN5500[31] have been successfully incorporated into polymeric micelles. We are currently conducting preclinical studies on these drug preparations. Kataoka *et al.* also succeeded in incorporating oligonucleotide into polyion complex micelles, and this suggests a possible application of the micellar system as a gene delivery system in the future.[32]

A phase I clinical trial of NK911 is currently under way to clarify its safety and pharmacokinetic profiles, in addition to evaluating its antitumor activity. This study may provide important clinical information for future clinical trials of the above-mentioned micelle carrier systems. It is emphasized here that drug carrier systems such as DXR liposomes and DXR micelles may have to be selected properly bearing in mind the tumor vascular characteristics.

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Case Report

Advanced renal cell carcinoma treated with granulocyte-macrophage colony-stimulating factor gene therapy: A clinical course of the first Japanese experience

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Abstract A phase I study of granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transduced tumor vaccine for patients with metastatic renal cell carcinoma (RCC) was initiated in 1998, as the first cancer gene therapy in Japan. The study is still ongoing, but the first patient is presented here as a case report. The patient was a 60-year-old man with Stage IV CRC with multiple lung metastases. After surgical resection of the tumor, autologous tumor cells were transduced and cultured to produce GM-CSF. The patient received a total of 2.2×10^8 gene-transduced autologous vaccine cells by subcutaneous injection. During the course of vaccination, growth of the largest metastatic mass slowed to some extent; however, multiple new lesions developed. About 1 month after the start of low-dose IL-2 therapy, rapid and remarkable regression in a large lung hilar metastatic mass was noticed. The patient died of progressive disease 7 months after the start of GM-CSF gene therapy. Careful histological examination by autopsy revealed that the responding mass was infiltrated by CD8 positive dominant T lymphocytes, and did not exhibit vasculitis or any other changes associated with active autoimmune disease.

Key words gene therapy, granulocyte-macrophage colony-stimulating factor, renal cell carcinoma, vaccine.

Introduction

Treatment options for metastatic renal cell carcinoma (RCC) are limited due to the lack of effective systemic therapy. According to preclinical animal studies, vaccination using autologous granulocyte-macrophage colony-stimulating factor (GM-CSF) transduced tumor cells is considered to be one of the challenging immunotherapies for RCC.[1] In April 1998, we submitted our preclinical data on cell processing and gene transfer to the Institute of Medical Science, University of Tokyo (IMSUT) Institutional Review Board. In August 1998, the Japanese government granted permission to initiate gene therapy. Within the same year, the phase I study of a GM-CSF gene-transduced tumor vaccine for patients with metastatic RCC began as the first cancer gene therapy in Japan.[2] The study is currently being conducted as a multi-institutional study organized by IMSUT. The present report describes the clinical course of the first patient who received the GM-CSF gene therapy.

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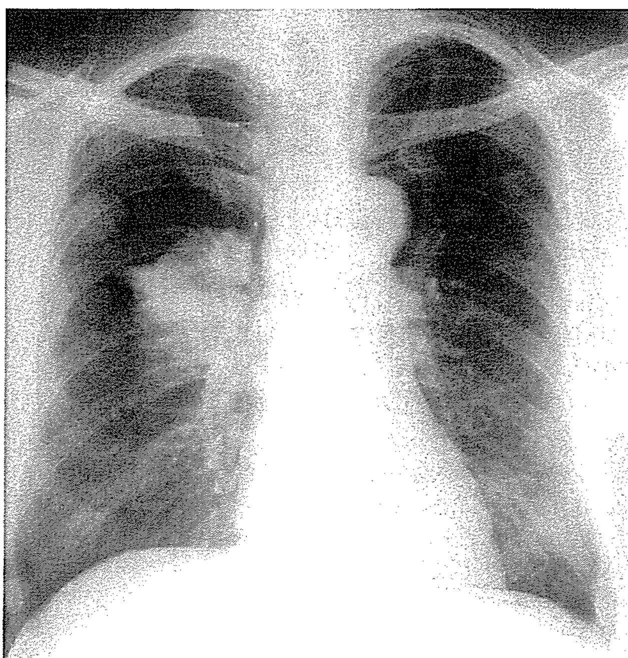

Fig. 1 Plain chest X-ray showing a large metastatic mass in the right hilar region and multiple metastases in both lungs.

Case report

Clinical course

A 60-year-old man with gross hematuria was seen at Tsukuba University Hospital (TUH) in September 1998. The patient was diagnosed as stage IV RCC (UICC classification 1997) with multiple lung metastases (Fig. 1). Following careful screening for eligibility criteria and after providing written informed consent, the patient was transferred to the Research Hospital of IMSUT, where he was enrolled in a phase I study of GM-CSF gene therapy. A 10 cm RCC tumor arising from the upper pole of the right kidney was resected, and the cell processing began on the same day. The largest part of resected tumor consisted of clear cell carcinoma with nuclear grade 2 as shown in Fig. 2a. The autologous RCC cell culture from 80 g of tumor tissue was successfully propagated, and the human GM-CSF gene was introduced using the MFG-S retrovirus vector.[1–4] Four-hundred and sixty million transduced cells with GM-CSF production of 49 ng/10^6 cells per 24 h were harvested. The harvested vaccine cells were lethally irradiated with 150 Gy of X-ray, then frozen in aliquots and stored in liquid nitrogen. BioReliance (Rockville, Maryland, USA) conducted standard safety testing according to US Food and Drug Administration guidelines and confirmed that culture supernatants were free of replication-competent retrovirus (RCR).

After safety and release testing, GM-CSF gene therapy was initiated by subcutaneous injection of vaccine cells. From December 1998 to April 1999, the patient was injected with a total of 2.2×10^8 vaccine cells divided into ten doses (4×10^7 cells in the first injection, followed by 2×10^7 cells at 2-week intervals). The patient experienced no remarkable side-effects except for low-grade fever, and local erythema and induration at the vaccination sites, both of which resolved spontaneously. Laboratory tests revealed no remarkable changes except transient eosinophilia up to 700 cells/mm^3, which peaked 48 h after vaccination and returned to normal the next day. There was no significant alteration in the serum GM-CSF level after vaccination. During the course of vaccination, the delayed-type hypersensitivity (DTH) reaction was examined by the intradermal injection of 1×10^6 lethally irradiated cultured autologous RCC cells (non-transduced RCC cells) as an in-vivo immunological examination.[3] As a control, the same number of lethally irradiated cultured autologous normal kidney cells, which were prepared from normal part of nephrectomy tissue, were injected intradermally. Reactions to DTH increased markedly after the start of vaccination; however, weak reaction was also observed at the site of normal kidney cell injection.

After therapy began, the growth of the largest lung hilar mass, as evaluated by helical computed tomography (CT), had slowed to some extent; however, new multiple nodules appeared in other lung fields. The patient maintained good performance status until the 10th injection, but routine radiographic examination at that time revealed development of new metastases in the liver and brain. At this point, vaccination was discontinued, and the patient was transferred to TUH for further treatment and supportive care. No RCR was detected in blood samples during or after the end of vaccination.

After transfer to TUH, asymptomatic brain metastases were treated with a single session of gamma-knife radiosurgery for prevention of central nervous system complications. Intravenous administration of human recombinant interleukin (IL)-2 (Teceleukin, Shionogi, Osaka), at a daily dose of 0.7–1.4×10^6 Japan Reference Unit (JRU), began 1 month after the end of the vaccination period. A few weeks after the start of IL-2 therapy, chest X-ray revealed a decrease in the size of the right hilar large mass, despite new metastatic deposits and pleural effusion in both lungs. The findings were confirmed by chest CT scan 1 month after the start of IL-2 therapy (Fig. 3). Treatment continued for 5 weeks