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Antitumor Effect of Implanted Ethylene–Vinyl Alcohol Copolymer Matrices Containing Anticancer Agents on Ehrlich Ascites Carcinoma and P388 Leukemia in Mice[1,2]

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Ethylene–vinyl alcohol (EVA1) copolymer was evaluated as a vehicle for controlled or sustained release of 5-fluorouracil (5-FU) and adriamycin. The variation of initial drug concentration and, to an even greater extent, the variation in comonomer ratio affected the drug release rate as well as the cumulative amount of the drug released. An increase in the ethylene content of the EVA1 copolymer decreased drug release. It appears that EVA1 copolymer can be used as a membrane for controlling the release of 5-FU or adriamycin.

The antitumor activity of EVA1 copolymer matrices containing these anticancer agents was evaluated against Ehrlich ascites carcinoma (EAC) in mice on the basis of changes in body weight and animal survival data. Tumor cell injections were performed on day 0 and matrix implantations on day 3, both intraperitoneally. The suppressive effect of matrices containing anticancer agents on the increase in body weight was higher than that of the free drugs. A prolongation of the life span of tumor-bearing mice following implantation of therapeutic matrices was also noted. Implantation of the EVA1 copolymer matrix containing an anticancer agent was less effective against the P388 leukemia than the EAC.

These results suggest that EVA1 copolymer matrices containing anticancer agents may be effective in cancer chemotherapy. Matrices composed of EVA1 copolymer could be useful vehicles for implanted delivery systems for anticancer agents.

Keywords——ethylene–vinyl alcohol copolymer; drug vehicle; drug delivery; controlled release; sustained release; 5-fluorouracil; adriamycin; Ehrlich ascites carcinoma; P388 leukemia; mice

The possible use of ethylene–vinyl alcohol (EVA1) copolymer as a new synthetic polymer for drug delivery has been examined.[4,5] The EVA1 copolymer is a flexible, heat-processable, and inexpensive material. The unique characteristic of this copolymer is its hydrophilicity.[6] The safety and biocompatibility of the copolymer are reflected in its use as hemodialysis membrane.[7] The EVA1 copolymer may be applicable as a unique drug carrier for implanted, inserted, or surface-applied devices because of its hydrophilic character.

In the field of cancer chemotherapy, topical administration of a controlled-release preparation into cancerous lesions has been attempted to maximize the effectiveness of anticancer agents and to minimize toxic side effects. The controlled-release preparations can deliver a steady quantity of drugs to a target area over long periods of time. Much of the previous work on controlled-release drug delivery has utilized silicone rubber for nitrosourea derivatives,[8,9] cytarabine arabinoside,[10] cyclophosphamide,[11] *etc.*

In the previous paper,[12] it was demonstrated that the release rate of a potent anticancer agent, 5-fluorouracil (5-FU), could be easily controlled by modifying the ethylene/vinyl

alcohol ratio in EVA1 copolymer matrices. Herein, we describe the antitumor activities of EVA1 copolymer matrices containing 5-FU against Ehrlich ascites carcinoma (EAC) and P388 leukemia in mice. We have also investigated the entrapment of adriamycin in EVA1 copolymers and the effect of the resulting matrices on transplanted tumors in mice.

Experimental

Materials——EVA1 copolymers with 43 and 54 mol% ethylene content were obtained from Kuraray Co. 5-FU and adriamycin were obtained from Sigma Chemical Co. and Kyowa Hakko Kogyo Co., respectively.

Preparation of EVA1 Copolymer Matrices——Controlled-release EVA1 copolymer matrices containing anticancer agents were prepared by the incorporation of the drug into EVA1 copolymers, as described previously.[12] The drug–copolymer matrices were fabricated in the shape of a 2.2 cm^2 disk, 1 cm in diameter and 0.2 cm in thickness (5-FU), and a 1×1 cm^2 square, 0.03 cm in thickness (adriamycin). The EVA1 copolymer and the required amount of drug were dissolved in the solvent (*n*-propyl alcohol : water = 3 : 1) at 80—85 °C. This mixture was poured onto a polyester film and the solvent was allowed to evaporate off. The residue obtained was melt-pressed to produce a disk or a membrane of uniform thickness. The drug content was calculated from the weight ratio of drug and copolymer used.

Measurement of Release Rate——The prepared controlled-release matrices were placed in vials containing 5 ml of distilled water (5-FU) or release medium (water : methanol = 1 : 1, adriamycin). The latter was kept in the dark. The drug release was followed by shaking the vials at a rate of 60 strokes/min on an incubator at 37 °C. At each time point, each matrix was successively transferred to a vials containing 5 ml of fresh release medium. The drug concentration of the samples was determined spectrophotometrically by measuring the absorption at 266 nm for 5-FU and at 233 nm for adriamycin. Release studies were done in triplicate and the average values were plotted.

Animals and Tumors——Male ddY mice (29—35 g) and hybrid BDF_1 mice (C57BL/6 × DBA/2, 18—24 g) were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. EAC and P388 leukemia were supplied by the Cancer Research Institute, Hokkaido University School of Medicine, and have been passaged at weekly intervals by intraperitoneal inoculation of ascites cells into ddY mice and DBA/2 mice, respectively.

Therapeutic Efficiencies on Tumor-Bearing Mice——In order to evaluate the antitumor effect of the EVA1 copolymer matrix containing anticancer agents, ddY mice and BDF_1 mice were inoculated intraperitoneally with 2×10^6 EAC cells and 2×10^6 P388 leukemia cells, respectively. One day (P388 leukemia) or three days (EAC) after inoculation of the cells, the mice were implanted with EVA1 copolymer matrix containing an anticancer agent or EVA1 copolymer matrix without any drug. The mice were anesthetized with pentobarbital and a small incision was made through the skin on the abdomen. The matrix was inserted intraperitoneally and pushed away from the incision area. The skin incision was sutured and the animals were placed in a cage. Other mice were injected with 0.3 ml of 5-FU suspension (free 5-FU) or 0.3 ml of adriamycin solution (free adriamycin) in saline. Changes in body weight and survival time of the tumor-bearing mice were recorded. The mice were observed for 60 d.

Results

In Vitro Release of Anticancer Agents from the EVA1 Copolymer Matrices

In this study, two sets of polymer–drug matrices were prepared and the release of anticancer agents dispersed in the matrices was investigated. The polymeric carrier in matrix A consists of a copolymer with 43 mol% ethylene comonomer content; in matrix B the ethylene comonomer content was 54 mol%.

Figures 1 and 2 show plots of the data, expressed as the cumulative amount of the drug released (Q) per unit surface area *versus* the square root of time ($t^{1/2}$),[13] for 5-FU and adriamycin, respectively. There appeared to be two phases: (a) an initial period of rapid release, termed the burst effect, due to the release of drug molecules at the matrix surface and (b) a period when release was approximately linear with respect to $t^{1/2}$. The initial-state (a) and steady-state (b) rates of drug release (k) were estimated from the slope of the linear Q–$t^{1/2}$ profile. The results are shown in Tables I and II for 5-Fu and adriamycin, respectively. An increase in the amount of ethylene comonomer content decreases the rate of drug release from these polymer composite matrices. It is known that the release rate decreases as the hydrophilicity of the polymer decreases,[14] and that the introduction of ethylene comonomer

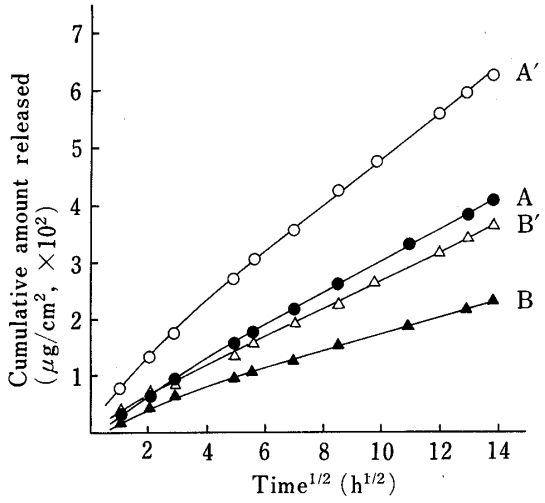

Fig. 1. Cumulative Amount of 5-FU Release from EVA1 Copolymer Matrices at 37 °C

A (3.9 mg matrix, ●) and A′ (7.2 mg matrix, ○), 43 mol% ethylene copolymer; B (3.9 mg matrix, ▲) and B′ (7.2 mg matrix, △), 54 mol% ethylene copolymer.

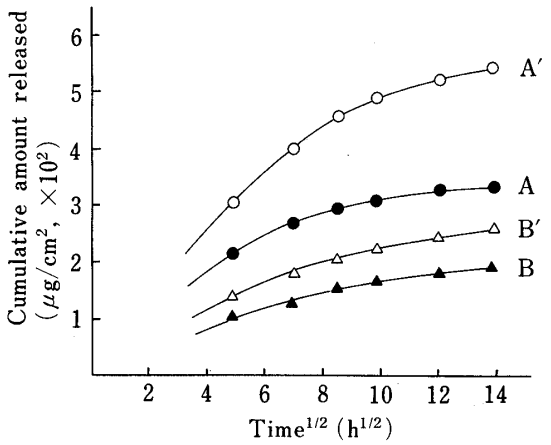

Fig. 2. Cumulative Amount of Adriamycin Release from EVA1 Copolymer Matrices at 37°C

A (1.3 mg matrix, ●) and A′ (2.6 mg matrix, ○), 43 mol% ethylene copolymer; B (1.3 mg matrix, ▲) and B′ (2.6 mg matrix, △), 54 mol% ethylene copolymer.

TABLE I. Effect of Ethylene Content of EVA1 Copolymer Matrices on Rate Constant of 5-FU Release

Matrix	Ethylene content (mol%)	Drug content (mg)	Release rate constant[a] (k, μg/cm^2/h$^{1/2}$)		Cumulative amount released[b] (mg)
			Initial state	Steady state	
A	43	3.9	32.99	27.69	0.91
A′	43	7.2	54.89	39.46	1.46
B	54	3.9	20.26	21.85	0.54
B′	54	7.2	26.71	25.22	0.80

a) The initial-state and steady-state rates of drug release (k) were estimated from the slope of the first 8 h of drug release and the slope of the linear Q–$t^{1/2}$ profile up to 8 d, respectively. *b*) The total amount of 5-FU released during the 8 d test period.

TABLE II. Effect of Ethylene Content of EVA1 Copolymer Matrices on Rate Constant of Adriamycin Release

Matrix	Ethylene content (mol%)	Drug content (mg)	Release rate constant[a] (k, μg/cm^2/h$^{1/2}$)		Cumulative amount released[b] (mg)
			Initial state	Steady state	
A	43	1.3	21.97	5.22	0.70
A′	43	2.6	41.50	14.48	1.17
B	54	1.3	12.41	6.34	0.43
B′	54	2.6	18.24	8.47	0.55

a) The initial-state and steady-state rates of drug release (k) were estimated from the slope of the first 3 d of drug release and the slope of the linear Q–$t^{1/2}$ profile up to 8 d, respectively. *b*) The total amount of adriamycin release during the 8 d test period.

decreases the hydrophilic nature of the polyvinylalcohol polymer.[12,15]

The effect of total drug content of a matrix on the drug release rate was next examined. Matrices A and B prepared with copolymers of different ethylene content were prepared with different drug contents; as shown in Tables I and II, at lower initial drug content, the release rate and the cumulative amount released are reduced for both 43 mol% ethylene–EVA1 and 54 mol% ethylene–EVA1 copolymer matrices.

The results of the present investigation indicate that with this system, a wide spectrum of release rates can be achieved by modifying the ethylene/vinyl alcohol ratio and the initial drug content in the matrices. Matrices composed of EVA1 copolymer could thus be useful vehicles for the controlled release of both 5-FU and adriamycin.

Antitumor Activity of the EVA1 Copolymer Matrices Containing Anticancer Agents against Ehrlich Ascites Carcinoma

The antitumor activity of EVA1 copolymer matrix containing 5-FU was evaluated against EAC in mice. EVA1 copolymer matrices were prepared from the copolymer of

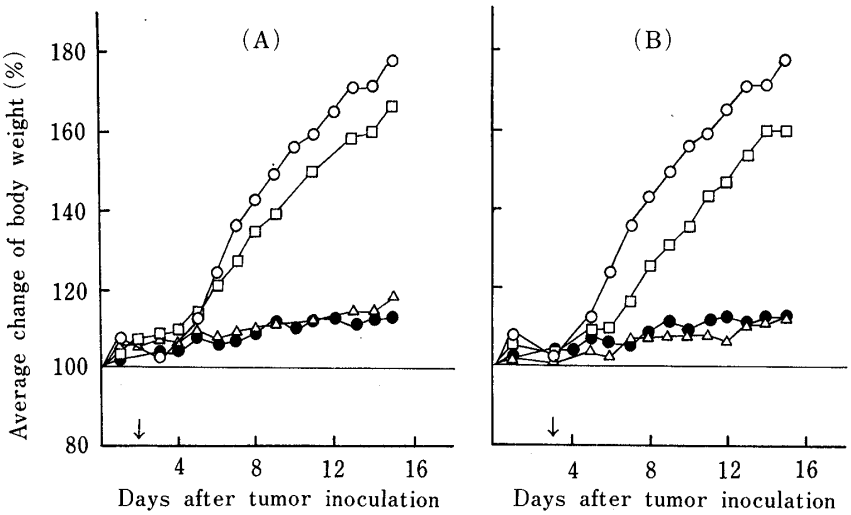

Fig. 3. Changes in Body Weight of Ehrlich Ascites Tumor-Bearing Mice after Implantation of Matrices Containing 3.9 (A) and 7.2 (B) mg of 5-FU per Matrix

—●—, control normal mice; —○—, control tumor-bearing mice; —□—, treated with free 5-FU; —△—, treated with EVA1 matrix containing 5-FU.
Arrows indicate the administration of chemotherapies.
In Fig. 3-A, chemotherapies were given at 2 d after inoculation.

TABLE III. Effect of EVA1 Copolymer Matrices Containing 5-FU and Free 5-FU on Survival Time of Mice Bearing Ehrlich Ascites Carcinoma

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	18.2±1.3	100.0	0/6
Free 5-FU	3.9[c]	22.2±1.6	122.0	0/6
	7.2	20.5±0.9	112.6	0/6
EVA1 matrix without 5-FU	—	20.7±0.7	113.7	0/6
EVA1 matrix containing 5-FU	3.9[c]	43.8±3.7[d]	240.7	1/6
	7.2	41.7±5.4[e]	229.1	0/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control. *c*) Chemotherapy was given at 2 d after inoculation. *d*) Significantly different from the control; $p<0.001$ and *e*) $p<0.005$.

43 mol% ethylene content. Tumor cell injections were performed on day 0 and matrix implantations on day 3, both intraperitoneally, unless otherwise noted.

First, the antitumor effect of EVA1 copolymer matrices was evaluated by following the changes in body weight. Figure 3 shows the changes in body weight in mice treated with free 5-FU and EVA1 copolymer matrix containing 5-FU. Figure 3 also shows the results in normal and tumor-bearing mice. At both doses, the increase in body weight afrer implantation of the matrices was smaller than that in the group receiving free 5-FU or in the control tumor-bearing group.

Next, the antitumor effect was evaluated on the basis of animal survival data. Table III summarizes the antitumor effects of free 5-FU, EVA1 copolymer matrices containing 5-FU, and EVA1 copolymer matrices without drug against EAC. All activities were calculated as $T/C\%$, the ratio of the mean survival time of the treated group (T) divided by that of the control group (C). T/C values greater than 125% indicate statistically significant antitumor effects.[16]

The mean survival time of the untreated control group was 18.2 d. Implantation of EVA1 copolymer matrices without drug did not produce any significant prolongation of survival time. When the mice were treated with 3.9 and 7.2 mg of free 5-FU, the mean survival times were 22.2 and 20.5 d and T/C values were 122.0 and 112.6%, respectively. In contrast, when EVA1 copolymer matrices containing 3.9 and 7.2 mg of 5-FU were implanted into the mice, the mean survival increased to 43.8 and 41.7 d and T/C was 240.7 and 229.1%, respectively. These values are significantly higher than those obtained with free 5-FU. One of six mice survived over 60 d after implantation of EVA1 copolymer matrices containing 3.9 mg of 5-FU.

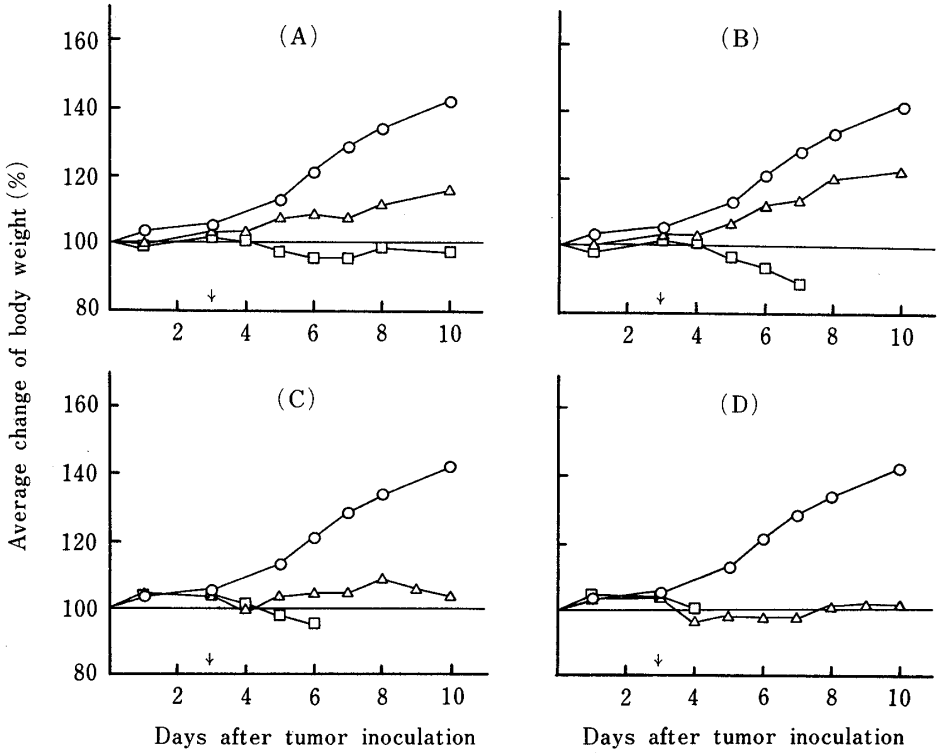

Fig. 4. Changes in Body Weight of Ehrlich Ascites Tumor-Bearing Mice after Implantation of Matrices Containing 0.3 (A), 0.7 (B), 1.3 (C), and 2.6 (D) mg of Adriamycin per Matrix

—○—, control tumor-bearing mice; —□—, treated with free adriamycin; —△—, treated with EVA1 matrix containing adriamycin.
Arrows indicate the administration of chemotherapies.

Table IV. Effect of EVA1 Copolymer Matrices Containing Adriamycin and Free Adriamycin on Survival Time of Mice Bearing Ehrlich Ascites Carcinoma

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	19.6±1.4	100.0	0/14
Free adriamycin	0.3	30.7±2.9[c]	156.6	0/7
	0.7	9.0±1.2	45.9	0/6
	1.3	6.5±0.2	33.2	0/6
	2.6	4.2±0.2	21.4	0/6
EVA1 matrix containing adriamycin	0.3	26.6±2.0[d]	135.7	0/7
	0.7	25.9±2.6[e]	132.1	0/7
	1.3	40.5±4.2[c]	206.6	0/6
	2.6	46.8±5.0[c]	238.6	2/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control. *c*) Significantly different from the control; $p<0.001$, *d*) $p<0.01$, and *e*) $p<0.05$.

Figure 4 shows the changes in body weight in mice treated with free adriamycin, EVA1 copolymer matrix containing adriamycin, and control tumor-bearing mice. Four levels of dose (0.3, 0.7, 1.3, and 2.6 mg per mouse) were used in this study. The increase in body weight after implantation of the copolymer matrices was smaller than that in tumor-bearing mice. In the group receiving free adriamycin, a decrease in body weight due to its toxicity was observed at all doses.

In Table IV, the antitumor activities of EVA1 copolymer matrices containing adriamycin and free adriamycin on EAC-bearing mice are summarized. Free adriamycin exhibited maximum antitumor activities on EAC at a dose of 0.3 mg/mouse, and T/C was 156.6%. Over this dose, free adriamycin exhibited a marked toxicity. On the other hand, EVA1 copolymer matrix had a slightly inferior activity as compared with free adriamycin at a dose of 0.3 mg/mouse, but above this dose, the EVA1 copolymer matrix exhibited marked antitumor activity and two of six mice bearing EAC were still surviving 60 d after receiving the drug at a dose of 2.6 mg.

Antitumor Activity of the EVA1 Copolymer Matrices Containing Anticancer Agents against P388 Leukemia

Antitumor activity of EVA1 copolymer matrices containing 5-FU or adriamycin was also evaluated against P388 leukemia in mice, based on survival data. P388 leukemia (2×10^6 cells) was inoculated intraperitoneally into BDF_1 mice. Chemotherapy was given with a single intraperitoneal injection (free drug) or implantation (EVA1 copolymer matrices) on day 1.

Table V shows the effect of the implanted EVA1 copolymer matrix containing 5-FU on the survival time of BDF_1 mice bearing P388 leukemia. The mean life span of the untreated control group with 2×10^6 P388 leukemia tumor cells was 9.7 d. Treatment with EVA1 copolymer matrices containing 3.9 and 7.2 mg of 5-FU increased the mean survival time, and the ratios of the mean survival time of the treated to control group (T/C%) were 142.3 and 132.0%, respectively. These results are significant, but are slightly smaller than those obtained with free 5-FU.

Table VI summarizes the antitumor activity of EVA1 copolymer matrix containing adriamycin against P388 leukemia in comparison with that of free adriamycin. Implantation of the EVA1 copolymer matrices containing 1.3 and 2.6 mg of adriamycin increased the T/C ratios to 110.3 and 142.3%, respectively, whereas free adriamycin showed marked toxicity at both doses.

Table V. Effect of EVA1 Copolymer Matrices Containing 5-FU and Free 5-FU on Survival Time of Mice Bearing P388 Leukemia

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	9.7±0.8	100.0	0/6
Free 5-FU	3.9	16.8±1.4[c]	173.2	0/6
	7.2	16.5±1.2[c]	170.1	0/6
EVA1 matrix containing 5-FU	3.9	13.8±0.7[d]	142.3	0/6
	7.2	12.8±0.7[e]	132.0	0/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control. *c*) Significantly different from the control; $p<0.001$, *d*) $p<0.005$, and *e*) $p<0.025$.

Table VI. Effect of EVA1 Copolymer Matrices Containing Adriamycin and Free Adriamycin on Survival Time of Mice Bearing P388 Leukemia

Compound	Dose (mg per mouse)	Survival time (d)[a]	T/C[b] (%)	Survivors at 60 d
Control	—	9.7±0.8	100.0	0/6
Free adriamycin	1.3	4.7±1.7	48.5	0/6
	2.6	2.0±0	20.6	0/6
EVA1 matrix containing adriamycin	1.3	10.7±2.8	110.3	0/6
	2.6	13.8±2.0[c]	142.3	0/6

a) Mean±S.E. *b*) Mean survival time of treated mice/mean survival time of control. *c*) Significantly different from the control; $p<0.1$.

Biocompatibility of EVA1 Copolymer

An EVA1 copolymer matrix without drug was prepared (43 mol% ethylene content) in the disk form and evaluated in terms of the number of survivors and evidence of rejection at 30 d after intraperitoneal implantation into normal ddY mice. None of the 7 mice examined died and no localized inflammation or foreign reaction in the peritoneum was observed. This copolymer did not induce significant fibrous encapsulation that could alter the drug release rate. The change in body weight after implantation of the pure EVA1 copolymer matrix was similar to that of normal untreated mice. In order to estimate the hydrophilic character of the copolymer, the water content of the copolymer matrix after implantation for 30 d was determined by the weight loss method.[12] The water content in the matrix was 7.0±0.5% (mean±S.E., $n=7$). Thus, EVA1 copolymer as an implanted carrier for drug delivery showed excellent biocompatibility.

Discussion

Adriamycin and 5-FU have been used extensively in the treatment of a variety of malignant diseases.[17] However, the clinical usefulness of these anticancer agents is severely restricted by the high toxicity.[18,19] Any dosage form or derivative of these anticancer agents with a lesser degree of toxicity or a higher chemotherapeutic efficiency should therefore be of great interest in the treatment of the various forms of leukemia and solid tumors.

Recently, much effort in cancer chemotherapy has been made to enhance the antitumor

effect of anticancer agents. One possible approach is the topical administration of a sustained-release preparation on cancerous lesions. As compared with conventional routes of drug administration, sustained-release systems that use implanted polymeric vehicles may have several advantages. In particular, they can deliver a steady quantity of drug to a target area over long periods of time. This method of local drug administration, in which the agents are delivered directly to the target tissues, may also minimize the systemic cytotoxicity. The procedure is convenient because it involves a single incision and insertion of a drug matrix rather than a repeated administration procedure.

The EVA1 copolymer has a hydrophilic character and good mechanical properties. It is a biomedical polymer prepared from biocompatible ethylene–vinyl acetate copolymer,[20] and has already been used as a hemodialysis membrane.[7] Further, our biocompatibility study of implants made of this copolymer showed no gross adverse effects at intraperitoneal sites in mice after a period of one month. The feasibility of using EVA1 copolymer as a polymer matrix for the controlled- or sustained-release of anticancer agents, 5-FU and adriamycin, is confirmed by this work.

The variation of initial drug concentration and, to an even greater extent, the variation of comonomer ratio affects both the drug release rate and the cumulative amount of drug released (Tables I and II). An increase in the ethylene content of the copolymer decreases its release property. This indicates that EVA1 copolymer can be used as a controlling membrane for the release of 5-FU or adriamycin. The desired release rate can be easily obtained by using EVA1 copolymer having a suitable ethylene content.

The EVA1 matrices containing 5-FU did limit the increase in body weight due to the tumor growth (Fig. 3) and bring about prolongation of the life span of EAC tumor-bearing mice (Table III). This result indicates that sustained drug release occurs in the peritoneum and that effective drug concentrations may be maintained by implantation of the EVA1 matrices. However, when BDF_1 mice bearing P388 leukemia were used, the mean survival time of the group receiving EVA1 copolymer matrix was significantly larger than that of the control group, though slightly smaller than that of the free 5-FU-treated group (Table V). Further work is necessary to identify the types of cancer that are amenable to such forms of drug administration and the types of drugs as well as the types of polymeric systems that have the potential of increasing the efficiency of drug use.

As is apparent in Table IV, the EVA1–adriamycin copolymer matrix was therapeutically more active at doses above 0.7 mg than adriamycin alone in the case of EAC, whereas at a dose of 0.3 mg the free drug was superior to the matrix. The high chemotherapeutic efficiency of the EVA1–adriamycin matrix was striking at high doses which would be toxic in the case of the free drug. This increased survival can be interpreted as a sign of reduced toxicity. The EVA1–adriamycin matrix dose appear to be less toxic than the equivalent amount of free adriamycin, if toxicity is evaluated in terms of the changes of body weight (Fig. 4).[21] LD_{50} of free adriamycin in mice was estimated to be 8—20 mg/kg,[22] but may be considerably higher when adriamycin is administered as the EVA1 copolymer matrix containing the drug. A prolongation in the life span of P388 leukemia-bearing mice following copolymer matrix implantation was also noted (Table VI). However, implantation of the EVA1–adriamycin matrix was less effective against the P388 leukemia than the EAC.

In conclusion, implantation of controlled- or sustained-release EVA1 copolymer matrices containing 5-FU or adriamycin can be an effective means of cancer chemotherapy. EVA1 copolymers show good biocompatibility and should be useful as a drug vehicle for implanted delivery systems of anticancer agents. Though this investigation was confined to experimental animals, the EVA1-anticancer agent matrix seems promising for clinical use. The technique should also be applicable to other chemotherapeutic agents, especially those that are hydrophilic in nature.[23]

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