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Early Phase Tumor Accumulation of Macromolecules: A Great Difference in Clearance Rate between Tumor and Normal Tissues

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The objective of this study was to investigate the molecular weight (MW) and time-dependence of the phenomenon termed "the enhanced permeability and retention" (EPR) effect in solid tumor, in particular to determine and define the early phase accumulation of macromolecules in tumor and normal tissues and the relationship between blood concentration and tissue clearance. As a model, radioiodinated *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers of MW ranging from 4.5 K to 800 K were administered i.v. to mice bearing sarcoma 180 tumor. Within 10 min all HPMA copolymers accumulated effectively in the tumor regardless of MW (1.0–1.5% of injected dose per g of tumor). However, higher MW copolymers (>50 K) showed significantly increased tumor accumulation after 6 h, while the lower MW copolymers (<40 K) were cleared rapidly from tumor tissue due to rapid diffusion back into the bloodstream. Blood clearance was also MW-dependent; the lower MW copolymers displayed rapid clearance, with kidney radioactivity of the copolymers of MW <20 K representing 24% of injected dose per g kidney at 1 min after i.v. administration. Within 10 min these copolymers passed through the kidney and were excreted in the urine. Higher MW copolymers consistently showed kidney levels of 3–5% dose per g kidney in the early phase with no time-dependent accumulation in kidney. There was also no progressive accumulation in muscle or liver, regardless of polymer MW. These results suggest the "EPR effect" in solid tumor primarily arises from in the difference in clearance rate between the solid tumor and the normal tissues after initial penetration of the polymers into these tissues.

Key words: Polymer drugs — Tumor targeting accumulation — EPR effect — Tumor vascular permeability — Tissue clearance

Soluble macromolecules such as albumin, poly(styrene-*co*-maleic acid-half-*n*-butylate) conjugated neocarzinostatin (SMANCS) and lipids such as the lipid contrast agent Lipiodol,[1–4] polymeric drug conjugates,[5] proteins[6] and liposomes[7, 8] exhibit enhanced or selective accumulation in solid tumors. This phenomenon has been termed "the enhanced permeability and retention (EPR) effect" by Maeda and colleagues[9–11] and is attributed to: (i) high vascular density of the tumor, (ii) increased permeability of tumor vessels, (iii) defective tumor vascular architecture, and (iv) defective or suppressed lymphatic drainage in the tumor interstitium. As the EPR effect seems of fundamental importance for tumor-selective targeting using macromolecular anticancer agents, there is considerable need to understand more clearly the mechanism of the EPR effect.

The use of various proteins with different molecular weights (MWs)[9] and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers of defined MW[12] administered either to mice, rats, or rabbits,[10, 11, 13] has shown clearly that there is a relationship between MW and time-dependent tumor accumulation. Molecules of higher MW progressively accumulate in a variety of solid tumor models, the phenomenon being most pronounced when nonbiodegradable polymers are used as probes. Bradykinin,[14, 15] nitric oxide[16, 17] and vascular permeability factors[18–20] have been shown to enhance vascular permeability.[21] However, it is still not clear whether it is the rate of tumor uptake of macromolecules or their tumor clearance which ultimately governs accumulation with time.

For this reason, we examined in the present study the early phase tissue uptake (focusing on the period within 10 min after administration) of ^{125}I-labeled HPMA copolymers of different MW and narrow polydispersity in mice bearing solid tumor. The late phase distribution of HPMA copolymers (>6 h) in tumor and normal tissues was also investigated in this study. HPMA copolymers provide a useful probe, as their MW-dependent body distribution is well characterized[22] and they have been used to prepare covalent conjugates of many anticancer agents including HPMA copolymer doxorubicin (PK1), which is now

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undergoing early clinical testing in the U.K.[23] This conjugate has been shown to exhibit the preferential tumor uptake (EPR) effect.[5, 24]

MATERIALS AND METHODS

Reagents Various purified HPMA copolymer samples with well characterized MW ranging from 4.5 K to 800 K with narrow polydispersity (MW/MN≤1.2) were prepared as described previously and designed to contain 1 mol% of tyrosine residue for later radioactive iodination.[25] The polydispersity (weight average MW/number average MW) of all the copolymers was calculated to be 1.2 by gel permeation chromatography using standard HPMA copolymers with defined MW.[12] The HPMA-copolymer fraction showed mean MW of 4.5 K, 16 K, 40 K, 80 K, 150 K, 300 K, 600 K, 800 K, respectively.[12] Their chemical structure is shown in Fig. 1. The content of tyrosine was assessed by UV absorption measurement.

Radioiodination of HPMA copolymers HPMA copolymers were radioiodolabeled using the chloramine T method with a kit of reagents from ICN Biomedicals Inc., Costa Mesa, CA, as described previously.[26] Free [^{125}I]iodide was removed by Sephadex G-10 column chromatography after iodination. The specific radioactivity of all HPMA copolymer fractions was approximately 2.5×10^8 dpm/mg. The labeled copolymers were immediately used for body distribution studies.

***In vivo* tumor model** Male ddY, 6-week-old mice, weighing 30–35 g, from SLC Inc., Shizuoka, were implanted subcutaneously with ~2×10^6 sarcoma 180 cells passaged as ascitic fluid of ascitic sarcoma 180. The tissue distribution study was performed on day 10–12 after tumor implantation, when the tumors were 7–10 mm in diameter, but contained no necrotic region.

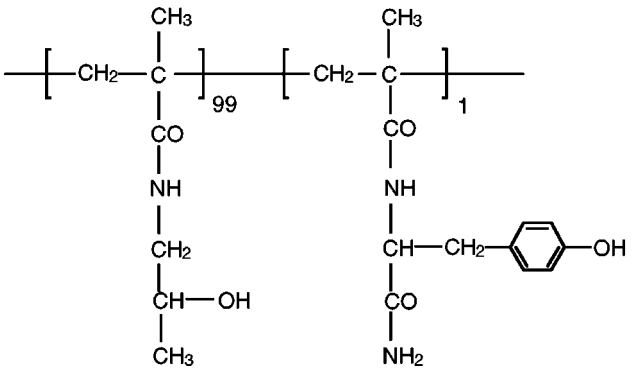

Fig. 1. Chemical structure of HPMA copolymers. Well defined copolymer fractions each having a narrow polydispersity were used in this study.

Body distribution and tumor accumulation studies ^{125}I-labeled HPMA copolymers were administered to mice via the tail vein (100 μg/ml/mouse). At various times after administration (1.0, 5.0, 10 min, 1 and 6 h) mice were killed and blood samples were drawn by cardiac puncture. Then, the mice were subjected to reperfusion with hep-

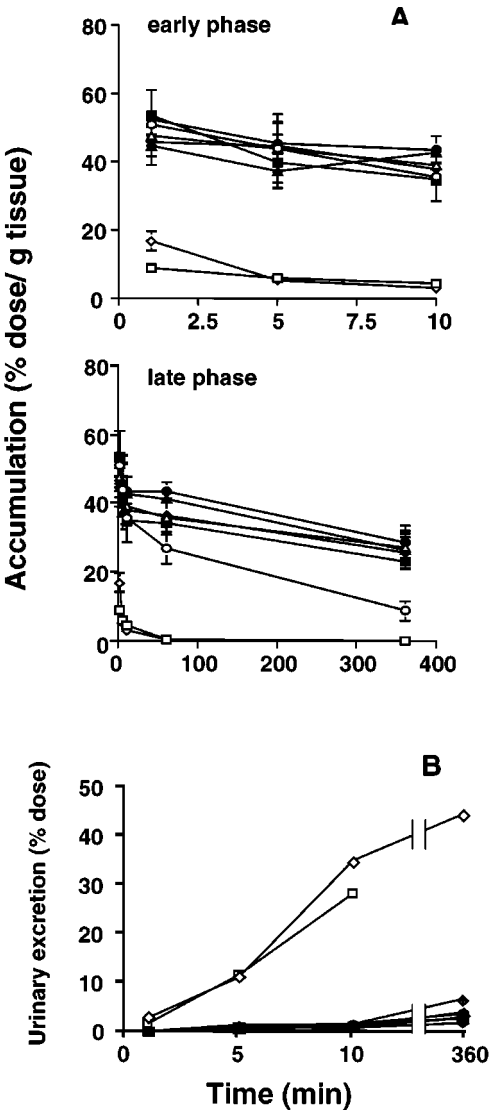

Fig. 2. Blood concentration and urinary excretion of ^{125}I-labeled macromolecular drug prototypes based on HPMA copolymer following i.v. administration to ddY mice bearing sarcoma 180 tumor. A, blood; B, urine. Three blood samples were taken at death, and each point represents the mean±SE (n=3). The concentrations of HPMA copolymers are based on radioactivity: MW: 800 K (▲); 600 K (●); 300 K (◆); 150 K (■); 80 K (△); 40 K (○); 16.2 K (◇); 4.5 K (□). Values are means±SE (n=3), except in B (n=2).

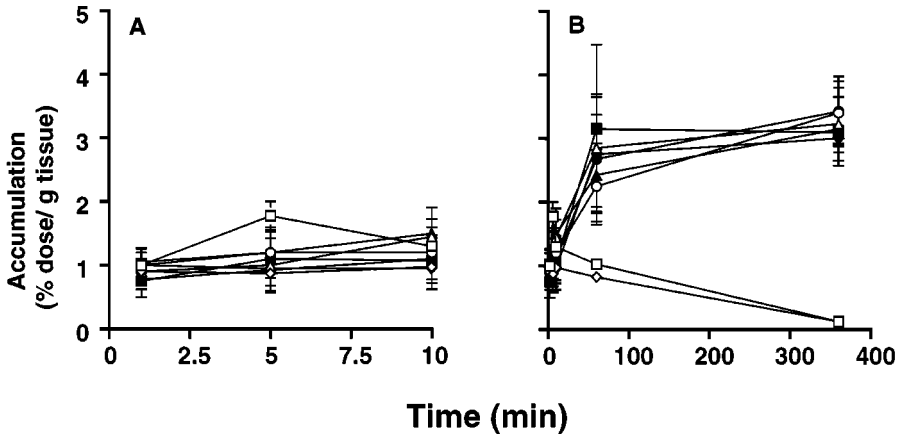

Fig. 3. Accumulation of ^{125}I-labeled HPMA copolymers in solid sarcoma 180 tumor tissues. A, the early phase; B, the late phase (longer time period); Symbols used for copolymers with different MW are the same as in Fig. 2.

arin-containing saline in order to remove blood components in the blood vessels of the tissues. Each specimen was removed and weighed, and the radioactivity was counted to estimate the total recovery of the dose administered, reflecting the amount of the polymer drug retained in the tissue interstitium and in cells.

Statistical analysis Student's *t* test was applied for the evaluation of *P* value.

RESULTS

Blood clearance and renal excretion of HPMA copolymers after i.v. administration Clearance of HPMA copolymers from the bloodstream showed a strong dependence on MW (Fig. 2, A and B). Both copolymer samples of MW<20 K (4.5 K and 16.2 K) had the same profile of blood clearance and urinary excretion rate; they were cleared rapidly from the bloodstream with an initial $t_{1/2}$ of less than 1 min. In contrast, the higher MW copolymers (MW≥80 K) remained in the bloodstream much longer, and after 6 h, 25–40% of injected dose/g blood still remained. At this time only 0.1% dose/g of the lower MW copolymers was detectable in the circulation. At 6 h, the sample with MW 40 K (closest to the renal excretion threshold for HPMA copolymer) showed a decrease in the blood level to almost half of its original value, although it was retained far longer than the lower MW copolymers of <20 K.

The renal excretion rates of the copolymers were compared at 5 and 10 min after i.v. administration (Fig. 2B). A great difference was seen between high and low MW copolymers, reflecting the renal excretion threshold.

Tumor accumulation of HPMA copolymers in early and late phase In the early phase, i.e., within 10 min of

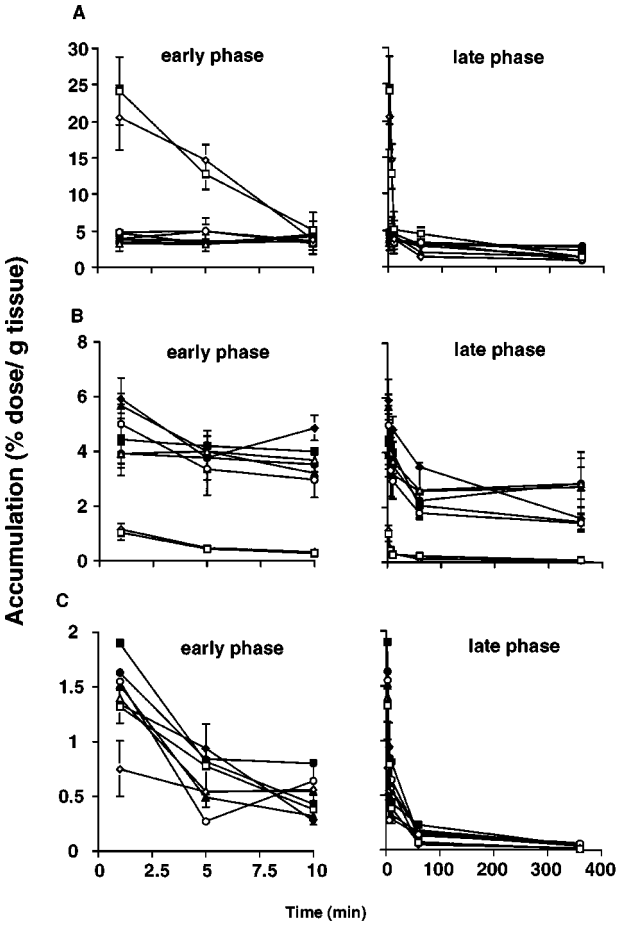

Fig. 4. Accumulation of ^{125}I-labeled HPMA copolymers in the kidney (A), the liver (B) and the muscle (C). Symbols used for copolymers with different MW are the same as in Fig. 2.

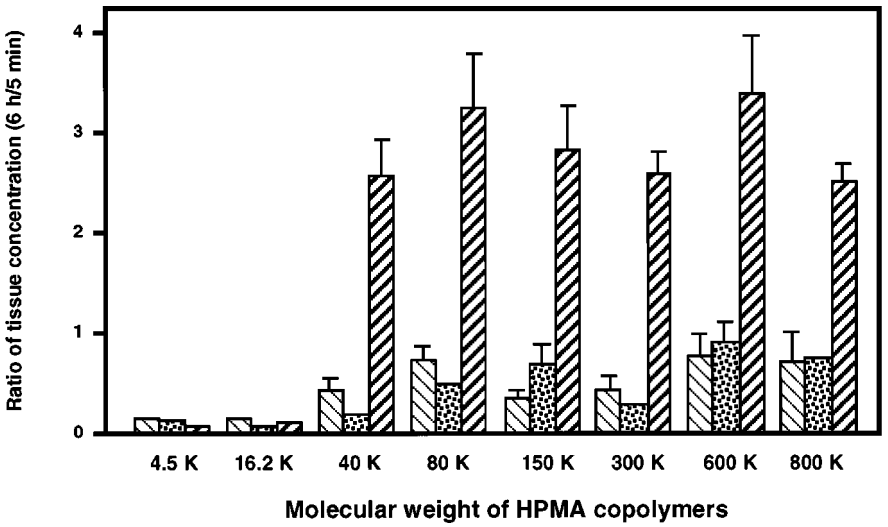

Fig. 5. Molecular weight-dependent accumulation in tumor and normal tissues of various molecular weight range polymers following i.v. administration to tumor (sarcoma 180)-bearing ddY mice. The values on the ordinate are the ratios of tissue-accumulation value (% dose) at 6 h divided by that at 5 min. Values are means±SE (n=3). Liver (▨), kidney (▩), and tumor (▧).

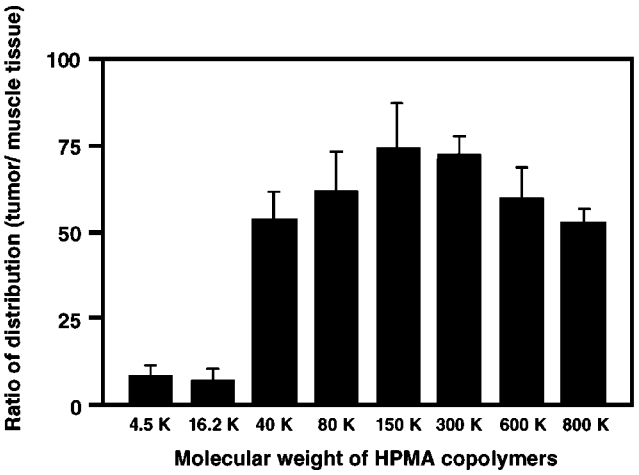

Fig. 6. Molecular weight-dependent tumor accumulation. Ratios of tumor- and normal-tissue (muscle) accumulation of various MW polymers following i.v. administration to tumor (sarcoma 180)-bearing ddY mice. The values on the ordinate are the ratios of calculated values of tumor accumulation at 6 h divided by that of muscle at 6 h. Values are means±SE (n=3).

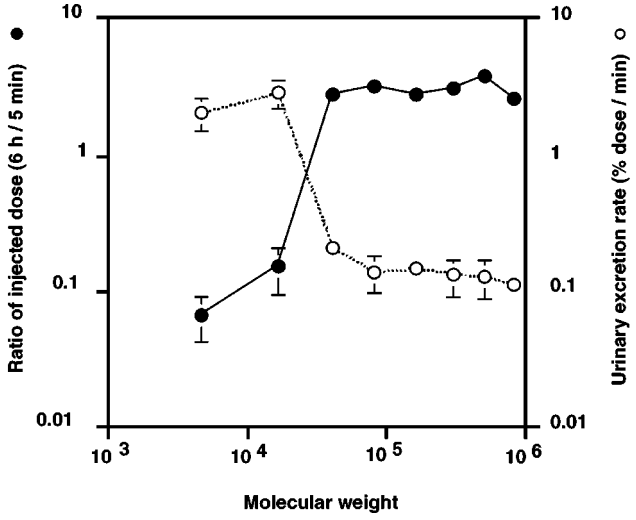

Fig. 7. Time-dependent accumulation of HPMA polymers of various MW in tumor following i.v. administration to ddY mice bearing sarcoma 180, and urinary excretion rates. For the time-dependent tumor-accumulation of HPMA copolymers, the ratio was calculated with tumor accumulation values (% of injected dose) at 6 h divided by those at 5 min (●). Urinary excretion rate (○) was calculated from the total urinary excretion values (% injected dose) at 5 and 10 min after administration.

administration, all HPMA copolymers showed significant accumulation in tumor tissue (~1% dose/g) without any MW dependency (Fig. 3A). Progressive tumor accumulation became apparent from 1 h after administration for the higher MW copolymers to levels of 2.5–3.0% dose/g (Figs. 3B, 5 and 6). In contrast, the lower MW copolymers showed little or no increase in tumor accumulation (0.7–1.1% dose/g).

After 6 h, the difference in tumor levels of high and low MW polymers became very marked (Figs. 3B, 5 and

6). In the case of the lower MW copolymers, as the plasma concentration decreased it appears that copolymer initially delivered into the tumor during the early phase, diffused back into the bloodstream and was then eliminated by renal excretion.

Normal tissue distribution of HPMA copolymers in the early phase The normal tissue distribution of HPMA copolymers was examined in kidney, liver and muscle (Fig. 4, A, B and C). Radioactivity counts in the kidney showed a strong inverse MW-dependency (Fig. 4A). At 1 min after i.v. administration of the copolymers of MW<20 K in the kidney values for recovered radioactivity were 20–24% of the injected dose/g. For the higher MW copolymers the corresponding values were 3–5%/g. In the early phase, within 10 min after administration, the kidney levels of radioactivity seen after administration of the lower MW copolymers decreased rapidly, whilst values detected for the higher MW copolymers remained consistently low. This is easily explained, as the copolymers of MW<20 K pass through the kidney, without tubular re-uptake and are quickly eliminated into the urine.

Levels of radioactivity detected in the liver showed the same pattern as the blood clearance profiles for all HPMA copolymers (Fig. 4B). However, an important observation is that none of the normal tissues studied showed evidence of time-dependent progressive increase in the accumulation of HPMA copolymers. The clearance of polymers in the liver, shown previously to be hepatobiliary transfer,[26] was so effective that the concentration of these polymers did not build up with time.

The levels of HPMA copolymers detected in the muscle were relatively high at the early phase and declined within 10 min. The copolymers were cleared very rapidly and their accumulation fell to a low level within 1 h with no subsequent time-dependent increase (Fig. 4C).

Time-dependent tumor accumulation of the higher MW HPMA copolymers in tumor tissue: the EPR effect Progressive time-dependent accumulation of HPMA copolymers in tumor tissue was compared with the changes in normal tissues: the kidney, the muscle and the liver (Fig. 5). Ratios of radioactivity (% dose/g tissue) of HPMA copolymer values in tumor observed at 6 h and 5 min after i.v. administration showed a clear-cut MW dependency (Figs. 5 and 7). The lower MW copolymers (<20 K) displayed a 6 h/5 min ratio of approximately 0.1. In contrast, the higher MW copolymers (≥40 K) exhibited ratios (6 h/5 min) of 2.7–3.1 in the tumor, whereas in the kidney and the liver, the ratios were 0.1–0.2 for the lower MW copolymers, and 0.3–1.0 for the higher MW copolymers.

Tumor levels of HPMA copolymers at 6 h after i.v. administration were compared with histologically similar orthotopic tissue, muscle, where the sarcoma 180 was inoculated (Fig. 6). The tumor/normal tissue ratios indicated a strong MW-dependency. Namely, in the muscle, the 6 h/5 min ratio was 7–8 for the lower MW copolymers, but 53–75 for the higher MW polymers. Furthermore, time-dependent progressive accumulation of the higher MW HPMA copolymers in tumor tissue was much more evident than in the normal tissues (Fig. 7). The initial urinary excretion rates of the higher MW copolymers were 0.11–0.13% dose/min, whilst those of the lower MW copolymers were 2.0–2.9% dose/min.

DISCUSSION

Improving tumor-selective drug delivery is a key objective in cancer chemotherapy using cytotoxic compounds. Macromolecular drugs, particularly polymer conjugates, are attracting increasing interest because of their passive and selective drug targeting to tumor tissue.[10–12, 27, 28] Examples of polymer conjugates under clinical study include SMANCS,[1, 3, 11, 29] which was approved and has been used for treatment of hepatoma in Japan since 1993, and HPMA copolymer-doxorubicin, designated PK-1, which is under phase I/II clinical evaluation in the U.K.[24] The EPR effect is not only observed in tumor tissue, but has also been described at sites of inflammation,[30, 31] i.e., water-soluble polymers such as poly(vinylalcohol), poly(ethylene glycol) and dextran with different MWs show preferred accumulation at the tumor and inflammatory sites.[10, 11, 27, 31]

Large polymers and small particles up to the size of bacteria can escape from the circulation via the EPR effect into tumor tissue in a number of different animal tumor models.[7, 9, 10, 13, 22, 32] This process clearly exhibits MW dependency, but it was not clear whether tumor uptake rate or clearance rate governs the observed molecular size dependency. Also, it was unclear whether the very early phase (immediately after i.v. administration) or the later phase is more susceptible to copolymer macromolecular weight effects. HPMA copolymers are loosely coiled neutral macromolecules that have no affinity for the plasma membranes or the blood vessels. Thus, they exist as free molecules and are believed to enter the cells by the mechanism of fluid-phase pinocytosis.[33] In the early phase after administration, it became very clear that the urinary excretion rate of HPMA copolymers is dependent on the MW of the copolymer (Figs. 2A/B and 7), and this explains why lower MW copolymers do not accumulate in normal tissue (Figs. 2–6). Retention of higher MW copolymers in the bloodstream is consistent with a size above the renal excretion threshold (~40 K), as reported previously.[9–11, 22]

The early phase tumor accumulation of all HPMA copolymers studied showed no MW-dependency (1.0–1.5%/g) (Fig. 3A), consistent with extravasation through the defective vascular architecture of tumor vessels[1–13, 31–36]

which may be under the influence of known vascular permeability factors such as vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF),[18–20] bradykinin (especially [hydroxypropyl^3] bradykinin),[14, 15, 21] and nitric oxide.[16, 17, 21]

Our present study confirmed the progressive time-dependent tumoritropic accumulation of higher MW HPMA copolymers in the late phase; at 1 h after administration of the copolymers one can observe the EPR effect in solid tumors, as reported earlier[1–4, 7–13, 16, 21, 27, 28, 30] (Fig. 3B). The tumor distributions of higher MW HPMA copolymers at 1 h showed 2- to 3-fold increases compared with that at 10 min after administration. This EPR effect contributes to the much slower clearance of those macromolecules from the tumor tissues or tumor interstitium into the lymphatics (Figs. 3 vs. 4–7), which is consistent with previous reports, including those on various lipids[1–4] and liposomes.[7] Very little clearance from the tumor tissue into the blood is observed for MW>40 K, i.e., above the renal excretion threshold (Fig. 7 and refs. 2, 4, 9), whereas clearance of small molecules *via* circulating blood is well known. The intratumor concentration remains high even after 100 h, even though the plasma level is very low.[9, 10] The copolymer concentration in the liver, which is a blood-rich normal tissue, more or less paralleled that in blood (Figs. 2A and 4B). Both present and previous studies showed that the normal tissues, i.e., liver, kidney, muscle, skin, etc., exhibit much faster clearance of these copolymers than tumor tissue, and no clear time-dependent accumulation was observed in these normal tissues, in contrast to the tumor tissues (Figs. 4 and 5). Although the present model used mouse sarcoma 180, we have found similar EPR effects in various animals and human tumors including B16 melanoma,[22] colon 38 and S-180,[9, 12, 13, 16, 21] and Ehrlich carcinoma (both unpublished data) in mice, Walker 256 carcinoma,[12] AH 136 and others in rats,[17–20, 22–24] rabbit VX2,[2, 4, 9, 10] and many human cancers in liver, lung, kidney, pancreas, etc.[1, 3, 10, 11]

The possibility exists that such higher level uptake of macromolecules and particulates in tumor tissue than in tnormal tissue could have resulted from uptake by reticuloendothelial cells, such as macrophages and polymorphonuclear cells, if such cells are abundantly found in solid tumor tissues. However, this seems unlikely. Wu *et al*.[7] demonstrated that stealth liposomes, which are coated with polyethyleneglycol and are not phagocytized by these cells and do not bind to vascular endothelia, were preferentially accumulated in tumor tissues. Therefore, the highly selective uptake of macromolecules, lipids and particulates in solid tumor tissues could be attributed to the EPR effect.

In conclusion, it was found that the early phase (<10 min) uptake rate into the tumor tissue is indiscriminately high for all molecular sizes of copolymers used (Fig. 3A), whereas the clearance rate either via blood or the lymphatics from the tumor was much faster for lower MW copolymers than for higher MW copolymers. Namely, macromolecular drug (>40 K) trafficking is predominantly one-way (blood $\rightarrow$ tumor), with little reverse flow (blood $\rightarrow$ tumor $\rightarrow$ blood $\rightarrow$ urine). This notion is also supported by the findings that low MW anticancer agents such as mitomycin C or doxorubicin injected into cavities (i.e., intraperitoneally or intrapleurally) are readily equilibrated with the blood level and are cleared rapidly, while high MW drugs such as SMANCS, or radioactive albumin or lipids (which behave like macromolecules *in vivo*) remain at high concentration for a long time [ref. 37 and Maeda, H. *et al*., unpublished data].

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