student, Ken Iwai, surgeon, MD from Konno's laboratory helped me this part of extensive experiments. Analysis of radioactivity in the tumor confirmed that the highest count—more than 2000-fold—occurred in the tumor compared with that in blood. Furthermore, in contrast to the usual situation for lipid particles in normal tissue, ^{14}C-labeled Lipiodol was not cleared from the tumor tissue as usually occurs via the lymphatic system [36,37]. Indeed, in lymphology, Lipiodol is used to visualize the lymphatic duct and lymph nodes via the X-ray system or by lipid-specific staining. Lipid particles or macromolecules are usually cleared or recovered by the lymphatic system from the tissue interstitium, and the fact that Lipiodol remained in the tumor suggested an impaired lymphatic clearance in the tumor tissues. Furthermore, the distribution of ^{14}C-labeled Lipiodol was only about a few percent of the injected doses in all normal organs such as the colon, kidney, and bone marrow, except for a relatively high value (10–15%) in the spleen and liver which were covered by the hepatic or splenic arteries near the injection route of hepatic artery. This finding indicates that this intraarterial method of SMANCS/Lipiodol administration would almost completely eliminate the systemic adverse effects caused by drug deposition in normal tissues, while producing an extremely effective antitumor effect because the tumor-selective drug accumulation was so marked. The high radioactivity counts of radio labeled lipid in the liver and spleen (normal tissues) were much reduced within a few days, perhaps because of normal lymphatic clearance. Other explanations for the higher radioactivity count in the liver may be the first-pass effect, because the artery, used for the injection, was the one supplying to the liver and the fact that lipid particles are usually recovered via the reticuloendothelial system of the liver and spleen.

Chapter 7

BACK TO THE BASICS IN INFECTION, INFLAMMATION, AND CANCER: THE ROLES OF PROTEASES, REACTIVE OXYGEN SPECIES (ROS), AND REACTIVE NITROGEN SPECIES (RNS) IN PATHOGENESIS

7.A. MICROBIAL DISEASE IN THE ABSENCE OF MICROORGANISMS: MICROBIAL PROTEASES AS PATHOGENS

In the Medical School, I was frequently asked to consult on various clinical problems, namely, important opportunities to interact with members of different clinical departments: Internal Medicine (I/II), Surgery (I/II), Ophthalmology, Dermatology, and Obstetrics-Gynecology, and others including departments in the basic sciences, such as Pharmacology, Anatomy and Pathology. Once, my colleague Professor Ryoichi Okamura, a professor of ophthalmology, showed me severe cases of corneal infection with *Serratia marcescens* and *Pseudomonas aeruginosa*, which did not respond to any antibiotics. The damage to the cornea was so severe and irreversible and the pain was so intense that some patients underwent extraction of the eyeball. Having some knowledge of bacterial proteases, I suggested that bacterial proteases could be the cause. In addition, Dr. Koki Matsumoto and Dr. Ryuji Kamata (who were then graduate students) came

to my laboratory to study whether serratia metalloproteinase triggered the kallikrein-kinin cascade, which we found, was initiated by activation of the Hageman factor (or Factor XII) and other factors [42-46]. Human humoral fluid contains no effective inhibitor of any of the proteases of *Serratia* or other bacteria. The end result is pain induced by bradykinin (or kinin) and induction of vascular extravasation of plasma proteins. We were able to measure the extent of vascular leakage of plasma proteins by injecting Evans blue (which bound to albumin in vivo) into guinea pigs; the dye would leak into the interstitial space (outside blood vessels). Then the amount of Evans blue was quantified after extraction from the skin or dermal tissue with formamide. (This method would become an invaluable tool in quantification of vascular leakage from solid tumors during the elucidation of the EPR effect, as described later.) We then found that many microbial proteases activated one or more steps in the kallikrein-kinin cascade, i.e., activation of the Hageman factor, prekallikrein or direct liberation of bradykinin from bradykinin from kininogen. Then, graduate student Akhteruzzaman Molla (who is now Director of the Virus Research Laboratory of Abbott Laboratories, Chicago), from Bangladesh, made significant contributions to this work, after Dr. Matsumoto completed his PhD degree.

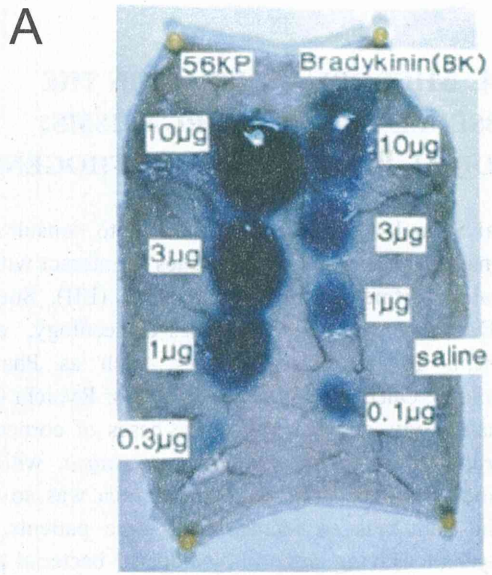


Figure 3. (Continued).

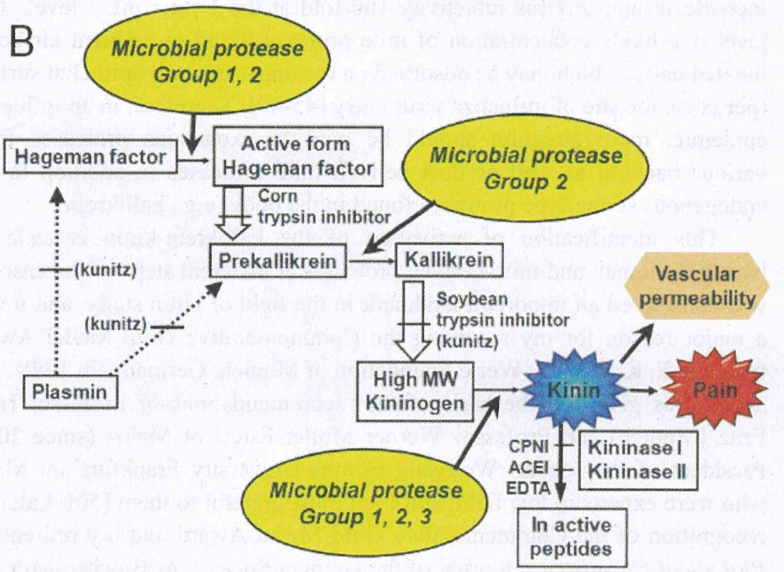


Figure 3. Bradykinin generation by bacterial proteases and activation of kallikrein-kinin cascade. (A) shows extravasation of Evans blue albumin complex induced by intradearmal injection of bradykinin (right) and serratial 56K protease (left). (B) shows kallikrein → kinin cascade and steps that microbial proteases activate. Kunitz, Kunitz type soybean trypsin inhibitor: Corn, corntrypsin inhibitor: CPNI, carboxy peptidase N inhibitor: ACEI, angiotensin conventing enzyme inhibitor: EDTA, ethylenediamine tetra acetic acid.

Extension of our research on bacterial proteases and house dust mite protease produced a surprising and intriguing finding: activation of influenza virus infectivity by cleavage of hemagglutinin on the surface of the virus. For this project, invaluable contributions of two graduate students—Takaaki Akaike and Keishi Maruo—should be mentioned [47,48]. Dr. Akaike (now, Professor of Microbiology, Kumamoto University Medical School) also investigated the true cause of viral pneumonia, as well as the molecular mechanism of double infection with both bacteria and influenza virus, in which proteases caused a more than 100-fold increase in viral infectivity. In our model, we administered, in addition to the virus, bacterial protease, at a dose of about 1 μg, as a bacterial effector. The reproduction of virus was indeed markedly enhanced by proteolytic cleavage of the hemagglutinin of influenza virus, a process that is required for virus infectivity. A surprising finding was that house dust mite protease, which commonly occurs in household air or in the environment of areas inhabited by humans, did

increase influenza virus infectivity 100-fold at the 1 $\mu g \cdot mL^{-1}$ level. This level is a likely concentration of mite protease found in ambient air being inhaled daily, which may be adsorbed on the upper tracheal epithelial surface (per cm^2), the site of influenza virus entry [45-49]. Therefore, in an influenza epidemic, more attention should be paid to exogenous proteases from various bacteria as well as dust derived mite proteases in addition to the endogenous serine-type proteases found in the body (e.g., kallikrein).

This identification of activation of the kallikrein-kinin cascade by bacterial, fungal, and mite-derived proteases at different steps of the cascade was considered an important landmark in the field of kinin study, and it was a major reason for my receiving the Commemorative Gold Medal Award from the E. K. Frey–E. Werle Foundation of Munich, Germany, in 1998. The award was given on the basis of the recommendations of Professor Hans Fritz (Munich) and Professor Werner Müller-Esterl of Mainz (since 2009, President of the Johann Wolfgang Goethe-University Frankfurt am Main) who were experts in this field, and I am most grateful to them [50]. Later, in recognition of the Commemorative Gold Medal Award and my retirement, *Biological Chemistry*, a journal of the German Society for Biochemistry and Molecular Biology produced a special issue (November, 2004). In 1995, the Japanese Society for Bacteriology had presented me with the Asakawa Award, its highest award, for the discovery of the pathogenic roles of bacterial proteases, particularly the finding of the mechanism of kinin generation and the involvement of proteases in the in vivo multiplication of influenza virus.

7.B. Microbial Disease in the Absence of Microorganisms: Endogenous Free Radicals as Pathogens

In my department during my chairmanship, we pursued various projects in multiple areas, including bacterial infection and proteases, viral infection and free radicals, and cancer chemotherapy and polymeric drugs. The interaction of different research fields yielded invaluable results, such as the role of polymer-conjugated superoxide dismutase (SOD) for the control of pathogenesis in influenza virus infection. This finding itself then led to the discovery of the superoxide anion radical ($O_2^{\bullet -}$) as a pathogenic molecule. It became the first demonstration of the occurrence of $O_2^{\bullet -}$ in viral disease in the absence of virus [51-53]. This concept went beyond the boundaries of the

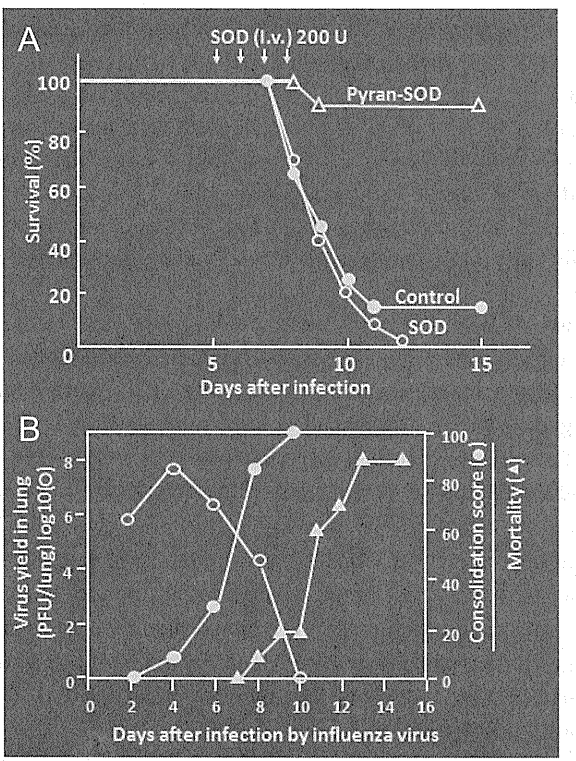
Postulates of Robert Koch, which required proof of the presence of defined microbial pathogens at the site of infection or in an ill subject [53, 54].

With reference to a different topic, viral infection triggers a number of events in host defense, or the immune response. One is the oxidative stress that is induced in influenza virus infection, which Dr. Linus Pauling first suggested. However, proving the presence of endogenous free radicals or ROS formation in animals and humans was quite difficult. If one removes ROS, and then as a consequence, one could at least successfully control pathogenesis by using an enzyme that removes superoxide radical; i.e., super oxide dismutase [SOD] which has a molecular size of 30 kDa. In working toward this goal of proving the presence of these radicals, I had enough experience with chemical modifications of NCS, and I knew that the in vivo half-life of small proteins such as SOD would be too short, that native SOD would not work when injected intravenously. Therefore, I designed an SOD to have a longer in vivo half-life by conjugating it with a biocompatible polymer, pyran copolymer [DIVEMA (divinyl ether and maleic anhydride copolymer)]. In collaboration with Dr. Takashi Hirano of Tsukuba, I prepared a pyran-SOD conjugate that had a more than 20-fold longer half-life in plasma after intravenous injection. Dr. Tatsuya Oda, my junior faculty, now Professor of Nagasaki University, and Akaike undertook experiment using influenza mouse model. Injecting this pyran-conjugated SOD into mice infected with influenza virus led to 98% survival, whereas almost all virus-infected control mice died 8–12 days after the infection began (Figure 4A).

It was interesting to follow the time course of viral yield in the lungs of infected control mice: the maximal virus yield was obtained on days 4–5, and from day 8 on mice started to die, but without detectable virus in the lung (Figure 4B). By day 12, all control mice had died, but we found no virus in their lungs (Figure 4A,B). This result means that the cause of viral death and the amount of virus found in the lung were unrelated. In contrast, when we scavenged the $O_2^{\bullet -}$ (or ROS) by means of pyran-SOD, the survival of mice improved greatly, which demonstrated the pathogenic role of $O_2^{\bullet -}$ in influenza virus infection (Figure 4A, B). Then the question was where and how $O_2^{\bullet -}$ generation took place? We then determined that the major source of $O_2^{\bullet -}$ generation was activation of xanthine oxidase in infected lung tissue of the mice (Figure 4C). These results were published in *Science* and other prestigious journal [51-53].

We successfully continued this line of research, and we later found that nitric oxide (NO) is generated in parallel with $O_2^{\bullet -}$ [55] (Figure 4C). Nitric oxide synthase (NOS) generates NO, mostly by the infiltrated leukocytes.

We then realized that $O_2^{\bullet -}$ and NO reacted extremely quickly, at a diffusion-rate manner limited to form peroxynitrite ($ONOO^-$) (Figure 5), which is highly oxidative and acts as a nitrating agent on proteins, nucleic acids, and lipids. We showed for the first time, in viral and bacterial infection models, that the nitration reaction and formation of mutants of virus and bacteria occurred at sites of infection (lesions) [55-57] (Figure 6). This nitration is ubiquitous and affects many aromatic molecules, such as tyrosine (to produce nitrotyrosine) and purines (nitroguanosine), as reported earlier. More interesting and more important, we demonstrated that nitration of guanine at the eighth position led to formation of 8-nitroguanine. 8-Nitroguanosine becomes a substrate of NOSs and cytochrome-P450 reductase etc, and then generate $O_2^{\bullet -}$, thus it lead to propagation reaction yielding $ONOO^-$ and nitration of G, then generation of $O_2^{\bullet -}$ from 8-nitroguanine [57].



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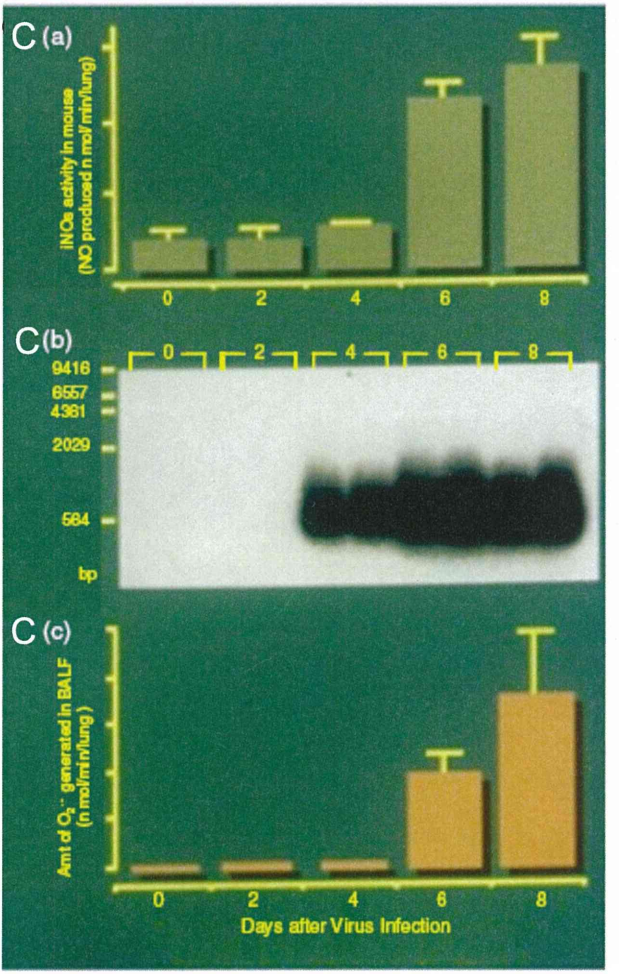

Figure 4. Influenza virus infection in mice. A. Effect of SOD on the survival of infected mice. SOD means mice injected with native SOD. SOD or pyran-conjugated SOD was injected intravenously (ref. [51-53,55]). B. Relationship between virus yield in the lung, consolidation score and mortality. C. (a) NOS activity, (b) amount of NOS in mRNA seen on agarose gel electrophoresis, and (c) generation of super oxide in the infected mouse lungs (see ref. [51-53, 55]).

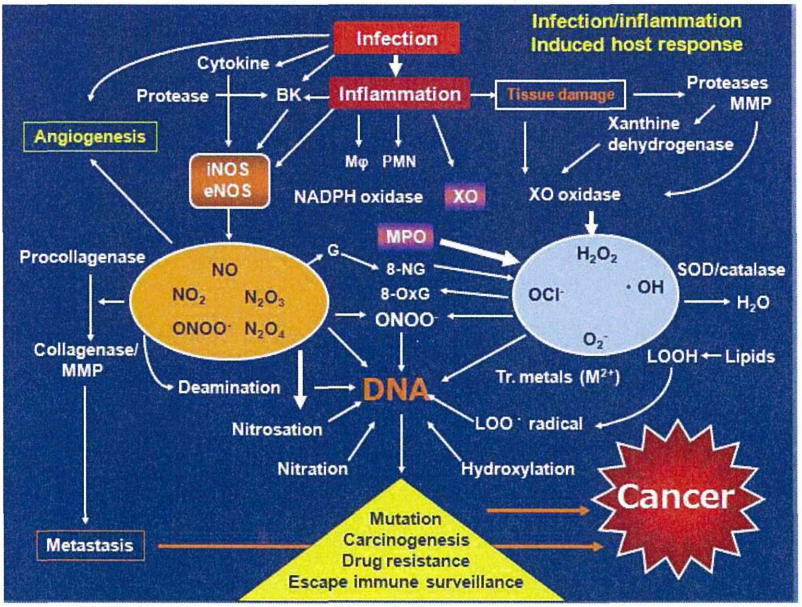

Figure 5. ROS and RNS in infection, inflammation and cancer and their interaction. Formation of $ONOO^-$ (peroxynitrite) and damage to DNA, or accelerates mutation and carcinogenesis should be noted.

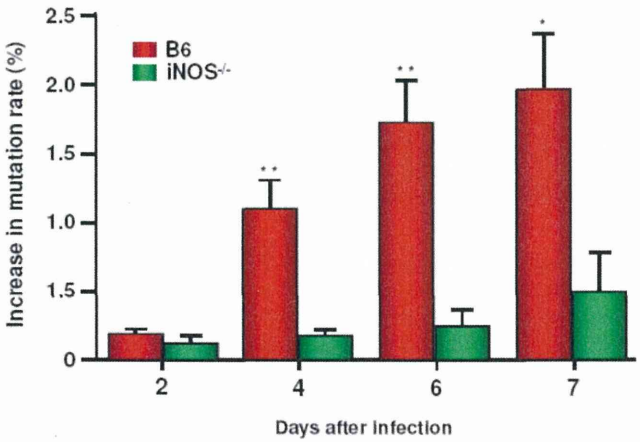

Figure 6. NO dependent increase of mutant virus in GFP-recombinant Sendai virus infected in wild mice (red bar) and not in iNOS knockout mice (green bar).

This result confirmed that the mechanism of mutation occurred at the site of infection, and consequently it also supported inflammation-induced carcinogenesis [54-59]. We reported that exposure to $ONOO^-$ caused marked (several-fold) acceleration of viral mutation [57,59-62] (Figure 6), as seen in influenza virus infection [55,56]. Dr. Hideo Kuwahara, a former student in our laboratory, had found $ONOO^-$ accelerated mutation in *Salmonella* seen by Ame's test , as well as drug-resistant mutants of *Helicobacter pylori* (at least a 7-fold higher frequency) in the presence of $ONOO^-$ at physiological and pathological concentrations [59,60]. Canolol, a phenolic compound that we found in crude rapeseed oil that scavenges $ONOO^-$ potently and also know high antioxidant activity, blocked this ROS/RNS-induced mutation [59,60,62], as well as gastric carcinogenesis produced by a carcinogen plus *H. pylori* infection in a *Mongolian gerbil* model, while it suppressed inflammation as revealed by collaboration with Dr. Shoei Tatematsu of Aichi Cancer Center of Nagoya [61]. More recently, Sawa et al reported formation of 3' 5' cyclic-8 nitroguanosine which appears to have a significant role in intracellular signaling pathway [63].

Chapter 8

CLARIFYING DETAILS OF THE EPR EFFECT: A UNIVERSAL SOLID TUMOR-TARGETING PRINCIPLE OF MACROMOLECULAR ANTICANCER AGENTS

At about the same time, in the late 1980s, Dr. Yasuhiro Matsumura, who originally trained as a surgeon and is now at the National Cancer Center Hospital East (head of Drug Development Division), Japan, joined our laboratory to pursue a PhD degree in cancer research. He played a critical role in identifying bradykinin (kinin) —as an effector responsible for facilitating extravasation to form ascetic fluid, in cancer patients (which occurs in carcinomatosis) [64]. This finding stimulated us to investigate the mechanism of such extravasation in cancer tissue in greater detail, because this enhanced vascular permeability was thought to sustain rapid tumor growth by supplying oxygen and nutrients, which may be regarded as the true cause of sustained growth of cancer cells. Moreover, we also thought to utilize this enhanced vascular permeability to control tumor growth, by suppressing kinin generation via either protease inhibitors or kinin antagonists.

My prior knowledge of the chemical modification of proteins was an important element leading to the discovery of the EPR (enhanced permeability and retention) effect [65]. Also, the method of quantitation of extravasation in infectious or inflammatory lesions as described earlier, could be readily applied to investigate extravasation more quantitatively in cancer tissue. Measuring Evans blue in tumor tissue could be viewed as

corresponding to measuring delivery of macromolecular drugs of about 67 kDa to tumor tissues, or to accumulation of such drugs in tumor tissues, which we did not see in normal tissues (Figure 7A-(1)). This observation was very intriguing. To validate such preferred accumulation of macromolecules in tumor tissue, we examined the effect of different molecular sizes on tumor uptake of macromolecules in a more detailed manner. The first series of experiments, carried out in mice, involved injecting radio labeled immunoglobulin (IgG, 160 kDa), transferrin (90 kDa), albumin (67 kDa), ovalbumin (47 kDa) and ovomucoid (28.8 kDa) from chicken egg white, and NCS (12 kDa). These substances were primarily labeled with diethylenetriaminepentaacetic acid (DTPA) (a chelating agent), followed by chelation of radioactive ^{51}Cr. The use of this labeling method was described earlier by Professor Claude F. Meares of the University of California, Davis, California. In SMANCS (16 kDa), the two free amino groups of NCS were blocked by conjugation with SMA, so no free amino group was available. Therefore, SMANCS was first cross-linked with free L-lysine via its free carboxyl groups to form amide linkage. DTPA was then added to the newly introduced amino group. With all these radioactive and biocompatible proteins in hand, Matsumura and I undertook extensive experiments. To my relief, my hypothesis that these natural proteins of more than 40 kDa would accumulate more selectively in cancer tissue was confirmed for all large proteins, but not for ovomucoids (< 30 kDa) and NCS, both of which are small proteins. Small proteins were quickly excreted into urine, without uptake by tumors. However, when SMANCS was bound to albumin, its behavior was more like that of true macromolecules, near 90 kDa (Figure 8 A,B).

We described these new findings in a manuscript that we submitted to the journal *Cancer Research* [65]. I believed that this intriguing phenomenon of extravasation and accumulation of macromolecules in tumors and their prolonged residence time in plasma would merit an attractive appellation. I therefore coined the designation ***"enhanced permeability and retention effect"*** of macromolecules in solid tumor tissues, or the EPR effect, which is now well accepted in the field of drug delivery, particularly in cancer targeting, for examples delivery of liposomes, micelles, antibodies, and DNA/RNA carrier complexes to tumors. The EPR effect is more than just a passive targeting of drugs, however, because its definition includes a prolonged period of drug retention in the tumor tissues. For instance, one can target any cancer drugs or imaging (contrast) agents to solid tumors if one injects them into a tumor-feeding artery; the drugs will be taken up more in tumor tissue but will disappear from the tumor within 5 min or so. This

passive targeting is in clear contrast to the EPR effect, because the passive targeting is a temporary phenomenon. In fact, radiologists know this targeting as a tumor stain seen in routine angiography. In our 1986 *Cancer Research* paper [65], we also reported that intratumor retention of Evans blue-albumin injected into tumor directly showed that the dye was far more persistent in tumor than retention in normal tissues. The normal tissues cleared the Evans blue-albumin within a few days or so, and the agent was almost completely gone by 1 week.

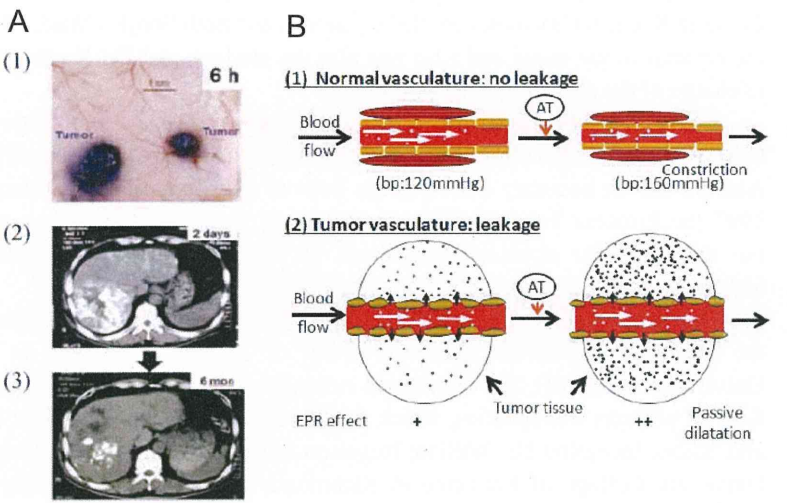

Figure 7. The EPR effect in a tumor in vivo (A) and a schematic illustration of an enhanced EPR effect under angiotensin-II (AT-II)-induced hypertension (B) (reproduced with permission from ref [66]. A: (1) S-180 tumors were implanted in the skin in mice, and when tumor reach to a palpable size Evans blue was injected intravenously. Dark blue spots in (1) demonstrate the tumor-selective accumulation of Evans blue-albumin; this means extravasation of Evans blue-albumin is observed as the EPR effect, which is not seen in normal tissue surrounding the tumors [65]. In Figure 7. A-(2) and -(3), CT scan images of primary hepatoma after SMANCS/Lipiodol injection via the hepatic artery which shows tumor selective drug (SMANCS/Lipidol) uptake by EPR effect in tumor as white area. CT scans were taken 2 days after administration and 6 months after (Figure 7A-(3)). B: The EPR effect does not occur in normal tissue but is observed in cancer tissue [65,66]. Under AT-II-induced hypertension, drug delivery to tumor can be further augmented, with more delivery of the macromolecular drug to tumor, as seen in Figure 7B-(2) on the right, which shows drug being pushed out more [67]. The method is now being applied in clinical settings [66].

The *Cancer Research* paper of 1986 was accepted by the two referees, one of whom wrote a comment directly on the manuscript: "Fantastic findings! Send to the press immediately! ". This paper, authored by Dr. Matsumura and I, was published very quickly, without any revision required. We were, of course, extremely excited. On only a few other occasions in my career did I have papers published in a first-class journal without revision needed. The paper was well received, as was the advent of clinical development of SMANCS. Around this period we were receiving great many hepatoma patients from all over Japan and also from abroad. Maki Clinic in Kikuchi City took care these patients, and both Shojiro Maki, M.D., the director of the clinic and who was also my student, and Dr. Konno were in charge of the clinical procedure.

Cured hapatoma patients from San Antonio, Texas and State of Oklahoma nominated me to become an honorary mayor of the City of San Antonio and an honorary citizen of the State of Oklahoma in 1989. Also, in 1997 the Princess Takamatsu Cancer Research Foundation in Tokyo gave me an award for academic excellence on the basis of development of SMANCS and discovery of the EPR effect in solid tumors.

One great supporter of the EPR effect was Professor Ruth Duncan (at the University of Keele, then University of London, UK, now at the University of Cardiff). She became an enthusiastic locomotive engine in the field of polymer therapeutics, which is now also called nanomedicine. She and others, including Dr. William Regelson of the Virginia Commonwealth University College of Medicine in Richmond, who had been working on DIVEMA (divinylether maleic anhydride/acid copolymer), also became good friends of mine during this time. Prof. Helmut Ringsdorf at University of Mainz, a renown polymer chemist and was a mentor of Ruth Duncan during her postdoc period, also became a strong supporter of EPR effect. Dr. Duncan and I coorganized a few international symposiums on polymer therapeutics in London and Japan. She sent me her young associate, Dr. Len Seymour (now Professor at the University of Oxford), to help continue investigations of the EPR effect and other developments in polymer therapeutics. I also received from her well-refined or discrete-sized copolymers of HPMA (hydroxypropyl methacrylate copolymer), which were prepared by the group led by Professor Karel Ulbrich of the Institute of Macromolecular Chemistry, in Prague, Czech Republic. After we radiolabeled these copolymers, we carefully examined their pharmacokinetics and retention in various tissues and tumors with a focus on their molecular size. We at Kumamoto also studied the kinetics of uptake of the copolymers by S-180 tumors, particularly at early time points (i.e.,

within 1–6 h) [68]; Dr. Duncan's group, then at the University of Birmingham, UK, performed similar studies with B16 melanoma [69]. Other research groups in Tsukuba, Tokyo, and Kyoto have confirmed the EPR effect in different tumor models. Use of HPMA-copolymer of discrete different molecular sizes to analyze the EPR effect thus provided more refined data [36, 68-71].

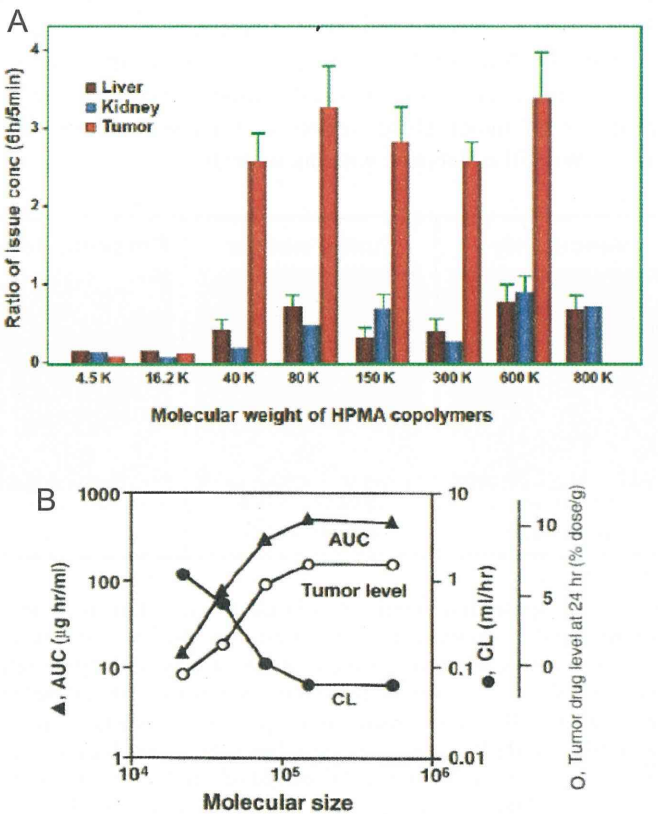

Figure 8. Relationship between the EPR effect and molecular weight of macromolecular drugs. Putative macromolecular drugs used were HPMA copolymers. A. Relative tissue uptake of these polymeric drugs in the liver, kidney and S-180 tumor at 6 hr was compared to that of 6 min after intravenous injection. MW and time dependent increase of tumor uptake is noted [68]. B. Relationship between molecular weight and AUC (area under the concentration curve of blood plasma), tumor uptake and renal clearance (CL). AUC in plasma paralleled to that in tumor [68,71]. A MW larger than 40KDa has a tendency of higher tumor retention.

Another researcher, the late Professor Judah Folkman (1933–2008) at Children's Hospital Boston and Harvard Medical School, also became a strong supporter of the EFR effect. His lifelong study of angiogenesis, tumor neovascularization, and vascular permeability in cancer tissue are integral components of the field of the vascular biology of tumors that he developed. The angiogenesis and EPR effect are the most crucial aspect of tumor growth in view of supplying oxygen and nutrients. I was also most impressed by the excellent scanning electron microscope images of vasculature of tumor blood vessels by Professor Paul O'Brien of the University of Melbourne then (now at Monash University Medical School), who reported leakage of polymers out of tumor blood vessels in *Cancer Research* in 1990 [72] (Figure 9). We still collaborate with his students.

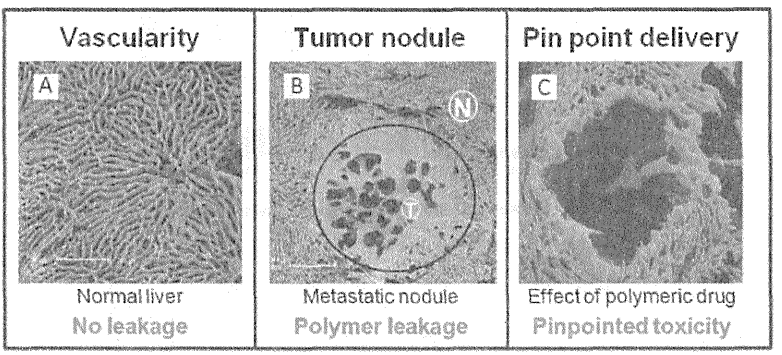

Figure 9. Scanning electron micrographs of vascular casts of plastic resin in the liver. (A) Normal capillary vasucullar structure of the liver. (B) Vascular structure of the liver with a metastatic microtumor nodule, as indicated by Ⓣ. Polymer resin extravasated only into the vascular bed of the tumor, whereas the normal vasculature Ⓝ did not show such polymer leakage. (C) In the same murine tumor model in mouse as that used for B, a macromolecular drug (SMA-pirarubicin micelles) had been injected i.v. 1 week earlier, and that caused selective disintegration of the tumor blood vascular bed (seen as an empty void, equivalent to the circled area containing polymeric resin in the encircled area of tumor in B). The tumor had been chemically induced by dimethylhydrazine in the colon of CBA mice, and a metastasis model was generated by injecting tumor cells into the spleen. Tumor-selective drug delivery and tumor selective damage could be achieved in the mouse even with a tumor size as small as 200 μm, i.e., micronodules. Tumor nodules as small as 200 μm already have unique blood vasculature, which is the evidence of tumor angiogenesis. These pictures are courtesy of Prof. C. Christophi and Ms. J. Daruwalla, University of Melbourne. Parts A and B are adapted with permission from ref. *84,127*.

Chapter 9

FACTORS FACILITATING THE EPR EFFECT

We had previously determined that bacterial infection generated bradykinin via activation of the kallikrein-kinin cascade, which is also a responsible factor for pain and edema formation and frequently accompanies tissue degeneration. The cause of this bradykinin-related pain and edema was identical in both inflammation and cancer. That is, this same mechanism may indeed function in cancer tissue, inasmuch as we had identified excessive bradykinin levels in ascitic and pleural tumor fluids [64,73,74]. These data were important because excessive bradykinin facilitates extravasation of fluid in the cavitary compartment and thus will play a role in ascitic and pleural fluid formation. Dr. Matsumura and Dr. Masami Kimura, who was then also a graduate student, in the Department of Surgery, clarified bradykinin formation in various cancerous ascitic and pleural fluids in many patients [73,74]. Inhibition of bradykinin formation by inhibiting kallikrein with soybean trypsin inhibitor suppressed ascitic fluid accumulation.

On the basis of these findings, I expected that activation of iNOS (the inducible form of NOS) and an accompanying NO generation would occur in tumor tissues. In one of our projects on infectious disease, we had developed a scavenger of NO, i.e., PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) [75]. Using PTIO, we found that NO was also involved in the EPR effect in solid tumors [76-79]. Then, Jun Wu (a graduate student from China, who is now at the City of Hope National Medical Center in California) studied various vascular mediators as well as antagonists and inhibitors of inflammation such as aspirin, indomethacin, kinin antagonists, and L-NMMA (L-*N*-monomethyl arginine) and confirmed

that these mediators affected the EPR effect as well [75-78]. Thus, the EPR effect occurred in cancer as well as in inflammation [73,74-79]. Among these mediators, collagenase and matrix metalloproteinase, activated by $ONOO^-$ (which derived from the reaction of NO with $O_2^{\bullet -}$), were also involved in the EPR effect [78,79]. Other factors that were found to facilitate the vascular permeability of tumors include tumor necrosis factor-α, vascular endothelial cell growth factor (VEGF), which was formally identified as vascular permeability factor by Dvorak and colleagues [80], and interleukin-12. I believe, however, that NO and bradykinin was the most important of all effectors. Therefore, the EPR effect is induced by multiple inflammatory factors (Table 1), and in fact the EPR effect is now demonstrated to function in tumors and inflammatory tissues similarly. Therefore, in terms of permeability and drug-delivery to disease site, various types of macromoleculer drugs or nanomedicines, including antibodies, DNA/RNA carrier complexes, polymer micelles and liposomes, the EPR effect is becoming crucially important, which is seen by the increase of citation numbers of the literature on the EPR effect published in recent ten years (Figure 10) (Table 2). During the preparation of this manuscript (Nov., 2010), we found carbon monoxide (CO) appeared another important factor for facilitating the EPR effect. CO is generated by the heme oxygenase (HO) during the heme catabolism that is carried out by an enzyme HO-1 (also called heat shock protein, Hsp-32), which is highly upregulated in most solod tumors and also in inflammatory state. Thus, HO-1 inhibitor is considered as a good therapeutic target [J. Fang and H. Maeda, in preparation].

Table 1. Factors affecting the EPR-effect of macromolecular drugs in solid tumor

Extensive production of vascular mediators that facilitate extravasation	
a) Bradykinin	b) Nitric oxide (NO)
c) VPF/VEGF	d) Prostaglandins
e) Collagenase (MMPs)	f) Peroxynitrite
g) Carbon monoxide (CO)	h) Inflammatory cells and H_2O_2
i) Tumor necrosis factor (TNF)-α	j) Anticancer agent

Table 2. Architectural differences and functions of tumor vasculature in comparison with normal vasculature

(1) Active angiogenesis and high vascular density
(2) Defective vascular architecture: ・Lack of smooth muscle layer ・Lack of or fewer receptors for angiotensin II ・Large gap in endothelial cell-cell junctions and fenestration ・Anomalous conformation of tumor vasculature (e.g.,branching or stretching)
(3) Defective lymphatic clearance of macromolecules and lipids from interstitial tissue (prolonged retention of these substances)
(4) Whimsical and bidirectional blood flow
(5) Macromolecular leakage (> 40 Kda upto size of bacteria) (ref. 65, 68, 71, 81, 82)

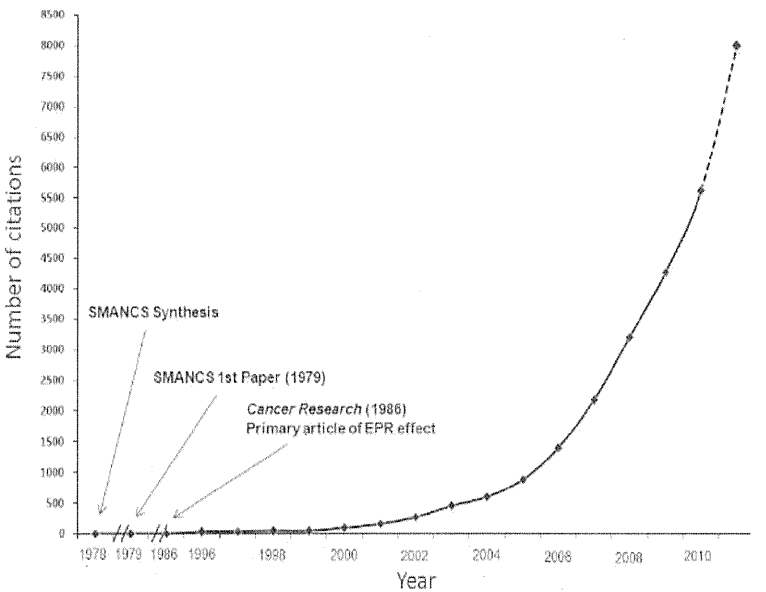

Figure 10. Citation frequency of papers on the EPR effect in solid tumors over time.

The Controlled Release Society awarded me in 2007 the Nagai Innovation Award for Outstanding Achievement on my contribution in the targeted anticancer drug delivery (EPR mechanism) and invention of SMANCS. This award is following after Robert Langer of MIT (2003) and Helmut Ringsdorf (2006). Detailed accounts of EPR effect have reviewed in many occasion [70,71,83-88,118-120]. Japan Society of Drug Delivery System also awarded me Nagai Award in June, 2011.