Expert Opinion

Inhibition of tumour metastasis by targeted delivery of antioxidant enzymes

Makiya Nishikawa† & Mitsuru Hashida
†*Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

Metastasis is one of the most harmful aspects of malignant neoplasm. Interaction of tumour cells with normal cells such as tissue macrophages may generate reactive oxygen species, which would affect various aspects of tumour metastasis. Reactive oxygen species cause damage to both tumour and normal cells and some of them, especially hydrogen peroxide, can also act as intracellular second messengers at sublethal concentrations to increase the transcription of various genes, which can then accelerate the proliferation of tumour cells in metastatic colonies. Therefore, eliminating hydrogen peroxide is one approach to inhibiting tumour metastasis. In this article, the roles of reactive oxygen species in tumour metastasis are reviewed, and the strategies to inhibit tumour metastasis by the targeted delivery of catalase, an enzyme that detoxifies hydrogen peroxide, are discussed.

Keywords: catalase, chemical modification, luciferase, metastasis, reactive oxygen species, targeted delivery

Expert Opin. Drug Deliv. (2006) 3(3):355-369

1. Introduction

Metastasis is not only the most characteristic aspect of malignant neoplasm, it is also the leading cause of death in cancer patients. Metastatic tumour growth or tumour recurrence can occur many years after surgical removal of the primary tumour. Unfortunately, the mechanism of tumour metastasis is not fully understood because of the complexity of the event, which makes its prevention or treatment difficult.

Tumour metastasis can be roughly divided into the following steps: tumour cell dissociation from the primary tumour; invasion of surrounding tissues; intravasation; distribution to distant organs; arrest in small vessels; adhesion to endothelial cells; extravasation; invasion of tissues in downstream organs and proliferation (Figure 1) [1,2]; as well as angiogenesis at the primary and secondary (metastatic) sites. Changes in the expression of various molecules, such as matrix metalloproteases (MMPs), adhesion molecules, growth factors and angiogenic factors, are associated with these processes in tumour metastasis. Generally speaking, tumour cells have abnormalities in their genes or in their gene expression profile. However, the changes in protein expression that are important for metastasis of tumour cells are attributed not only to tumour cells but also to the surrounding normal cells. Interaction of tumour cells with other cells, as well as the changes in the physiological conditions that are induced by the interaction, such as the embolisation of capillaries followed by reperfusion, can be stimuli that activate the expression of various molecules and so accelerating the metastasis. When tumour cells enter the systemic circulation, they bind to capillary endothelial cells through interactions of various molecules on the membranes of both tumour and endothelial cells. The specificity of the interaction, if it occurs, explains the tissue specificity of tumour metastasis [3]. An initial interaction between tumour cells and endothelial cells will activate both types of the cells through cytokines, free radicals, bioactive lipids and growth factors, leading to the

informa healthcare

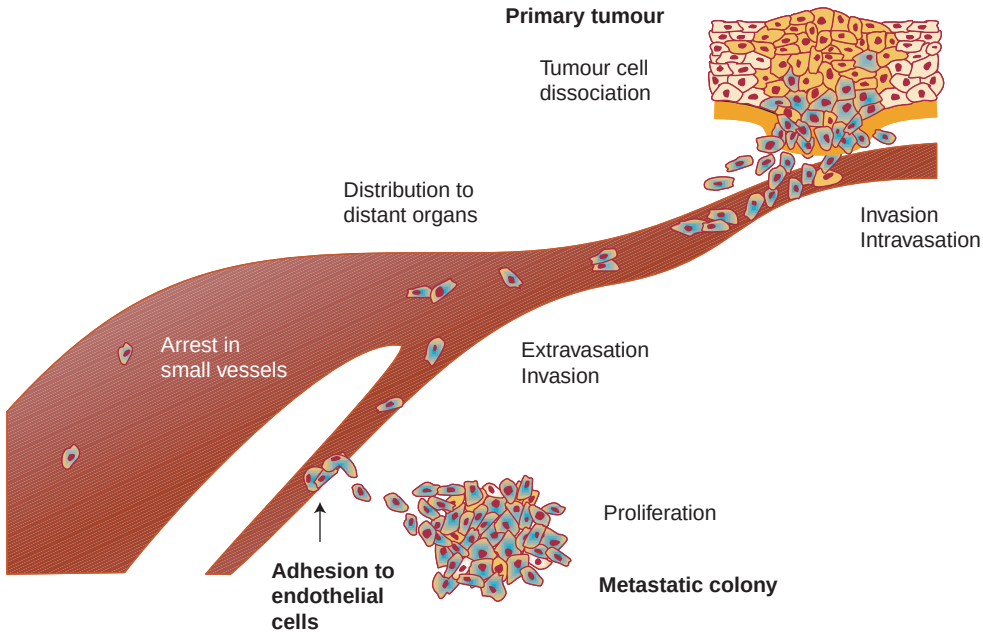

Figure 1. Steps in tumour metastasis. Tumour cells that dissociate from a primary tumour invade the surrounding tissues by degrading the extracellular matrix using several types of MMPs. After the breakdown of the basement membranes, they enter the blood or lymphatic circulation. The cells are distributed and are trapped by capillaries in downstream organs. The majority of the cells are destroyed during these processes but the few surviving are bound to endothelial cells and then extravasate via degrading basement membranes. After migrating into the extracellular matrix with the help of MMPs, tumour cells proliferate to form metastatic colonies. Several types of cells other than the tumour cells are involved in the establishment of tumour metastasis. Reprinted from NISHIKAWA M, HYOUDOU K, KOBAYASHI Y, UMEYAMA Y, TAKAKURA Y, HASHIDA M: Inhibition of metastatic tumour growth by targeted delivery of antioxidant enzymes. *J. Control. Rel.* (2005) **109**(1-3):101-107 [2]. Copyright (2005), with permission from Elsevier.
MMP: Marix metalloprotease.

increased expression of adhesion molecules that strengthen the initial adhesive bonds [4]. Thus, the metastasis of tumour cells is significantly affected by their interaction with other cells and this is, therefore, one reason why it is difficult to develop antimetastatic pharmaceuticals.

Of the many molecules that are involved in the pathogenesis of tumour metastasis, reactive oxygen species (ROS) play important roles in a variety of ways. ROS are used in various biological processes within the body, and their level is strictly regulated by the ROS-generating systems and the ROS-detoxifying systems (Figure 2). ROS are constantly generated in aerobic organisms during normal metabolism and in response to both internal and external stimuli. It has been proposed that imbalances in the production and removal of ROS play a causative role in a variety of disease pathologies, such as carcinogenesis, ischaemia/reperfusion injury and degenerative diseases, such as photoaging, atherosclerosis, arthritis and neurodegeneration. When the body is invaded by microbes or other potentially harmful materials, ROS are used to kill the invaders. At the same time, ROS are growth signals and a reduction in their level may result in a reduced proliferation.

When blood flow that is temporarily occluded by tumour cells is re-established by clearance of the cells, ROS and reactive nitrogen species are generated by ischaemia/reperfusion [5]. Oxidative stress that is induced by the injury and mechanical deformation may kill almost all of the tumour cells [6-8]. If a fraction of the tumour cells survive these deadly assaults, they then have greater opportunities to form metastatic nodules due to the proliferative nature of ROS. Therefore, tumour metastasis may be inhibited by either increasing or decreasing the ROS level in the vicinity of tumour cells. Recently, Laurent *et al.* reported that tumour cells are more susceptible to an increased level of hydrogen peroxide than normal cells, suggesting the usefulness of the ROS-generating system for cancer therapy [9]. On the other hand, the removal of ROS has been proved in several experimental settings to be effective in inhibiting tumour metastasis (see Section 4). In addition, tumour cells are considered to be under increased oxidative stress, due to oncogenic transformation, alterations in metabolic activity and increased production of ROS [10-12]. Although several approaches to generate ROS have been investigated for killing tumour cells, a gradient in the concentration of ROS may produce a situation where the proliferation or invasion of tumour cells, as well as angiogenesis, is increased by sublethal levels of ROS in primary and metastatic tumours.

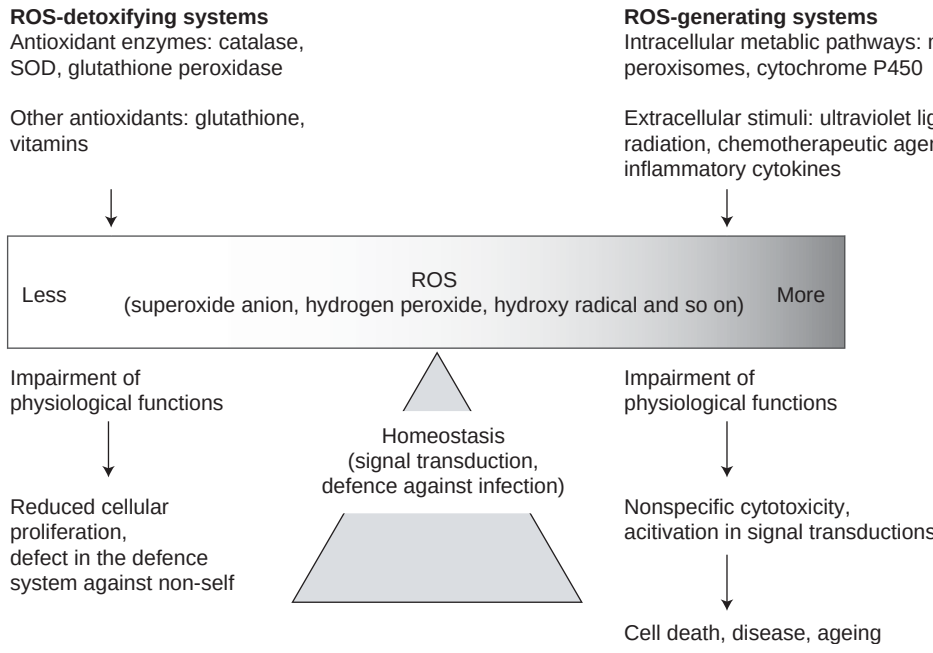

Figure 2. ROS and their functions. The ROS level within the body is strictly regulated by ROS-generating systems and ROS-detoxifying systems. When the level becomes low, biological functions may be affected: cellular proliferation may be reduced and there may be defects in the defence against non-self. On the other hand, a high level of ROS will also destroy biological functions. The cytotoxic nature of ROS may lead to cellular and tissue damage, leading to several types of diseases and various phenotypes of ageing.
ROS: Reactive oxygen species; SOD: Superoxide dismutase.

2. Involvement of reactive oxygen species in tumour metastasis

ROS play various roles in the processes of tumour metastasis. Activated phagocytes produce very high levels of ROS and many other types of cells also generate low levels of ROS [10,13,14]. Monocytes that are directly stimulated by tumour cells produce ROS [15]. In addition, stimulation with cytokines such as TNF-α or IL-1 will enhance ROS production in various cells [16]. When the blood flow is blocked due to the embolisation of tumour cells, the reperfusion of the flow generates large amounts of ROS. These molecules can be toxic to both normal and tumour cells. At the same time, they serve as subcellular messengers in gene-regulatory and signal transduction pathways; several molecules that are involved in tumour metastasis can be upregulated by ROS. Endogenous ROS-scavenging systems are sometimes overwhelmed by an increased level of ROS.

2.1 Cytotoxic nature of reactive oxygen species

ROS are powerful oxidants that are toxic to cells and cause cellular and tissue damage at high concentrations [17]. Metastasising tumour cells suffer from increased oxidative stress after entering the systemic circulation, from which most tumour cells are severely damaged, resulting in cell death. When injected into the tail vein of mice, only a small percentage of melanoma cells survived the first 24 h [18]. It has been reported that reoxygenation of hepatic cells after ischaemia may produce toxic species, including ROS, that lead to an elimination of weakly metastatic colorectal carcinoma cells within 24 h of their arrival in the liver [7].

ROS can be generated in various ways that are related to carcinogenesis or the therapeutic options that are chosen to treat cancer [19], such as radiation, ultraviolet light and anticancer agents. For example, radiation produces large amounts of singlet oxygen molecules, one of the most powerful oxidants known, which kill both tumour and normal cells. Ultraviolet irradiation increases the intracellular oxidative stress and induces apoptotic biochemical changes, such as DNA fragmentation and caspase-3 activation [20]. Doxorubicin, an anthracycline, reacts with CYP reductase to form a semiquinone-radical intermediate, which in turn reacts with oxygen to generate a superoxide anion radical and hydrogen peroxide [21]. Other anticancer agents, such as bleomycin and cisplatin, also generate ROS, which play an important role in drug-induced apoptosis [22,23]. ROS-generating enzymes have also been investigated as a possible antitumour treatment. Xanthine oxidase, which generates a superoxide anion radical and hydrogen peroxide, is delivered to solid tumours by conjugation with PEG, and combination with the administration of hypoxanthine (a substrate of xanthine oxidase) has been shown to produce significant suppression of tumour growth [24].

2.2 Reactive oxygen species as second messengers

Most tumour cells are destroyed during metastatic processes due to mechanical and oxidative stress. However, if a few survive and are able to proliferate, this is enough to form metastatic nodules that eventually kill the host.

Tumour cells that survive ROS attack will be influenced by the ROS. They are known to influence the expression of a number of genes and signal transduction pathways [25-27]. Most of the changes that are induced by ROS would be mediated by the mitogen-activated protein kinase and NFκB signalling pathways [27] as these contain multiple steps that are sensitive to ROS.

Exposure of normal and transformed cells to ultraviolet radiation or to hydrogen peroxide stimulates increased expression of proto-oncogenes, such as *jun-B*, *jun-D*, *c-fos* and *fos-B*. Expression of collagenases including MMPs [28,29], adhesion molecules [30], EGFR [31] and VEGF [32], all of which are highly associated with the metastatic nature of tumour cells, is also reported to be increased or activated by the presence of ROS. Nelson *et al.* reported that MMP expression is increased by elevated manganese superoxide dismutase (SOD) activity, suggesting the involvement of hydrogen peroxide in regulating metastasis [33]. Transendothelial migration, which is highly correlated with intravasation, of human melanoma cells is reported to be partly mediated by ROS-sensitive cellular signalling cascades [34]. One of the most important properties of ROS on tumour metastasis is their role in the stimulation of cell proliferation [14,35]. Overexpression of NADPH oxidase (Nox)-1 in NIH3T3 fibroblasts increases superoxide generation and cell proliferation, both of which return to normal levels after co-expression of catalase [36].

3. Controlling tissue distribution of antioxidant enzymes by chemical modification

Although catalase, SOD and other antioxidant enzymes are potentially effective in preventing tumour metastasis by degrading the ROS that would activate the transcription of various proteins that are associated with the event, inhibition of tumour metastasis by these enzymes can be achieved only when they are targeted to the sites where the level of ROS is increased. Generally speaking, clinical applications of biologically active proteins have had difficulty in achieving significant therapeutic effects, mainly due to problems associated with their pharmacokinetic properties. For instance, SOD has a very short plasma half-life following intravenous injection (only 5 – 10 min in rodents), and catalase from bovine liver is rapidly cleared by the liver after intravenous injection in mice [37]. Therefore, the tissue distribution of these enzymes needs to be controlled in order to inhibit tumour metastasis by degrading ROS.

Based on the relationship between the physicochemical and biological properties of macromolecules and the anatomical and physiological properties of tissues and organs, site-specific delivery of macromolecules has been successfully demonstrated [38,39]. To control the tissue distribution of bovine liver catalase for the inhibition of tumour metastasis, the authors of this review have used a variety of chemical modifications: galactosylation, mannosylation, succinylation and conjugation of PEG, or pegylation (Figure 3). In each case examined, the enzymatic activity remained at ≥ 90% of the original value [40] and this is an important parameter for evaluating whether any chemical modification can be used for targeted delivery of proteins or not. In addition, attention needs to be paid to the immunogenicity of the chemically modified proteins. In the following section, targeted delivery approaches are reviewed, irrespective of whether they have ever been applied to antioxidant enzymes. Because ligand-, antibody- or other unique structure-mediated interactions with body components will affect the tissue distribution of macromolecules, any approach that is developed for different macromolecular compounds can be applied to antioxidant enzymes.

3.1 Tissue distribution of macromolecules

The tissue distribution of a compound is a consequence of a myriad of interactions of the compound with various components of the body. Macromolecules, which include proteins, polysaccharides, oligo- and polynucleic acids and synthetic polymers, have a limited ability to pass through the capillary endothelium and the plasma membrane of cells. When macromolecules are injected into the extracellular space of tissues, such as skeletal muscle and solid tumours, the distribution within and outside the tissue is significantly limited compared with that of low molecular weight compounds [41-43]; the distribution also depends on the physicochemical properties of the molecule, such as its size and electric charge.

The tissue distribution of antioxidant enzymes and other macromolecules is a function of the physicochemical properties of the molecules and the anatomical and physiological properties of the body [44]. Distribution of macromolecules from capillaries to tissues is restricted by the capillary endothelium in most tissues except for the liver, spleen, bone marrow and kidneys, all of which have fenestrated or discontinuous capillaries [45]. The majority of the solid tumours also have vessels through which macromolecules can be easily transported. Because of these limitations in transport, macromolecules only distribute to some restricted organs and tissues with discontinuous or fenestrated endothelia, or to solid tumours [38,44,46]. These unique properties of macromolecules, as far as tissue distribution is concerned, have been used to deliver a variety of drugs to tissues such as solid tumours by conjugating them with macromolecules.

A major process determining the tissue distribution of macromolecules is glomerular filtration. Because this is a size- and charge-dependent process, the physicochemical properties of proteins largely affect the rate of filtration. The dependence of glomerular filtration on the size and charge has been examined in detail by using dextrans of varying size and charge [47]. Neutral dextrans with a molecular radius of ≥ 42 Å undergo

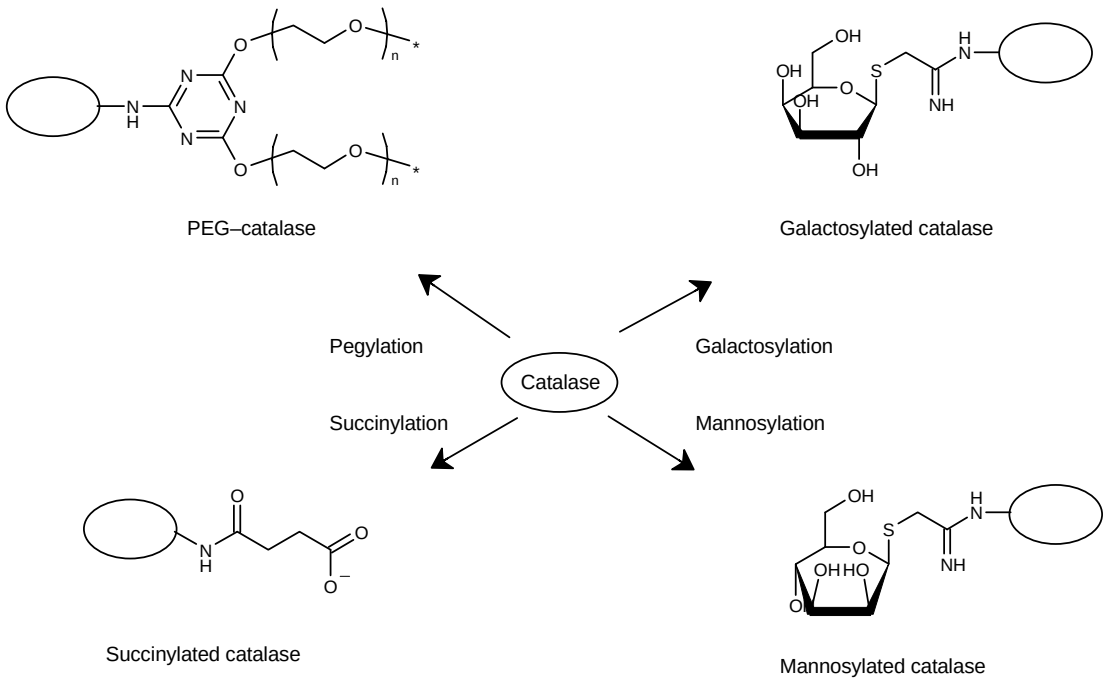

Figure 3. Chemical modification of catalase for controlling its tissue distribution. Bovine liver catalase can be modified with 2-imino-2-methoxyethyl 1-thiogalactoside (galactosylated catalase), 2-imino-2-methoxyethyl 1-thiomannoside (mannosylated catalase), succinic anhydride (succinylated catalase) or 2,4-bis(*O*-methoxypolyethylene-glycol)-6-chloro-*s*-triazine (PEG–catalase). Reprinted from NISHIKAWA M, HYOUDOU K, KOBAYASHI Y, UMEYAMA Y, TAKAKURA Y, HASHIDA M: Inhibition of metastatic tumour growth by targeted delivery of antioxidant enzymes. *J. Control. Rel.* (2005) **109**(1-3):101-107 [2]. Copyright (2005), with permission from Elsevier.
PEG: Poly(ethylene glycol).

hardly any filtration through the glomerulus, whereas smaller ones are filtered at a rate that increases as the molecular radius decreases. Comparing derivatives with similar molecular weights, negatively charged dextrans exhibited a lower filtration rate than those positively charged, indicating that the repulsion and attraction of charged molecules is involved in the filtration process. Serum albumin, the most abundant protein in the blood, has a negative charge at a physiological pH and a molecular weight (67,000 Da) that is greater than the threshold of filtration; it undergoes very little glomerular filtration under normal conditions; and circulates in the blood for a long time. In complete contrast to this, proteins with an effective molecular radius that is smaller than the threshold are filtered in the kidneys and disappear from the circulation at a rate that depends on their molecular weight. Using proteins with a variety of physicochemical properties, Nishikawa and colleagues demonstrated that recombinant human Cu^{2+}, Zn^{2+}-SOD (molecular weight of 32,000 Da), soybean trypsin inhibitor (20,100 Da) and chicken egg lysozyme (14,300 Da) have short half-lifes in the plasma following intravenous injection into mice [48]. Studies using isolated rat perfused kidneys have shown that the filtration of proteins is determined largely by their size [49]. Catalase, a protein with a high molecular weight of ~ 220,000, undergoes hardly any glomerular filtration.

When macromolecules undergo little filtration through the kidney glomeruli, they have a great opportunity to distribute to various sites of the body. The uptake by cells, including tissue macrophages, sinusoidal endothelial cells in the liver and hepatocytes, can be a factor that determines their tissue distribution. Various receptors, membrane proteins and glycoconjugates are known to be involved in the cellular uptake of macromolecules. These include carbohydrate receptors, such as asialoglycoprotein receptors, mannose receptors, fucose receptors, scavenger receptors and negatively charged sugar moieties (e.g., the sialic acid of sugar chains of glycoproteins and glycolipids). The negatively charged sugar moieties are present on the vast majority of cells and allow the binding of cationic macromolecules.

Kupffer cells in the liver and splenic macrophages also contribute to the tissue distribution of macromolecules circulating in the blood. They capture various macromolecules by sensing signals through receptors on the cell surface. Scavenger receptors are a class of receptors that recognise negatively charged molecules and they play important roles in the tissue distribution of macromolecules with strong negative charges, including acetylated low-density lipoproteins, oxidised low-density lipoprotein and DNA. Serum albumin is also cleared through these receptors when it ages to form advanced

glycation end products [50]. Chemically modified proteins are also ligands for these scavenger receptors when they possess strong negative charges [51].

Under pathological conditions, the anatomical and physiological properties of tissues may change, which will result in the altered tissue distribution of macromolecules. Inflammation will increase the permeability of the endothelium, which allows large molecules to extravasate and accumulate in the interstitial space of the inflamed tissues [52]. In addition, the properties can be intentionally altered by several means. Osmotic opening, which uses intracarotid infusion of a hypertonic arabinose or mannitol solution, has been proposed as a method to deliver hydrophilic compounds into the brain across the blood–brain barrier [53]. Vasodilatation and shrinkage of cerebrovascular endothelial cells, with widening of the interendothelial tight junctions to an estimated radius of 200 Å, can be induced following such an infusion. Injection of a large volume of saline into the tail vein of mice at a high velocity, the so-called hydrodynamics-based procedure [54], has become a standard method of achieving a high degree of transgene expression in the mouse liver by intravenous injection of naked plasmid DNA [55]. Although plasmid DNA is a huge macromolecule (≥ 4,000,000 Da) with a strong negative charge, it can be delivered by this method to the nucleus of hepatocytes without adversely affecting structural integrity, demonstrating that the barriers to the distribution of macromolecules are efficiently overcome by this method. Transient hyperpermeability of the hepatic cellular membrane [56] and the generation of membrane pores in hepatocytes [57] have been reported after large-volume injection into mice. The transport of naked plasmid DNA across the continuous capillaries in skeletal muscle has also been greatly improved by a similar large-volume injection method [58].

Because the tissue distribution of macromolecules is determined by the interaction of the molecules with biological components and the interaction is a function of the physicochemical properties of the molecules, these properties are important factors in determining their tissue distribution. Indeed, the size, electric charge and hydrophilic/lipophilic balance of macromolecules have been shown to have profound effects on their tissue distribution. This is the basis of the targeted delivery of proteins and other pharmaceutical compounds by controlling the overall physicochemical properties in order to obtain an improved therapeutic effect [44,59,60].

3.2 Increase in the circulation half-life

Glomerular filtration and removal by mononuclear phagocytes are the two main processes that shorten the plasma half-life of proteins. Both processes are effectively blocked by conjugation of water-soluble, inert macromolecules with proteins. PEG is the most frequently used, and probably most effective, polymer for this purpose [61,62]. Several protein–PEG conjugates are undergoing clinical trials and some have already been approved for clinical use. PEG-IFN-α_{2a} was approved by the FDA in 2002 for the management of chronic hepatitis C virus infection. Pegaspargase, a conjugate of L-asparaginase with PEG, is used for the treatment of various leukaemias. Conjugation of PEG, or pegylation, significantly increases the effective molecular radius of proteins. When proteins that undergo glomerular filtration are pegylated, such as SOD, the plasma half-life is greatly increased. Various types of PEG, such as linear, branched and comb-shaped forms, are available for protein conjugation, whereas other hydrophilic polymers are also used to increase the plasma half-life of proteins by conjugation. SOD has been conjugated with the pyran copolymer, carboxymethyl- and diethylaminoethyl-dextrans, and poly(vinylpyrrolidone-co-dimethyl maleic anhydride) copolymer. These SOD conjugates showed tissue distribution properties reflecting the properties of the polymer used for conjugation. Gregoriadis *et al.* developed various protein conjugates using colominic acid, a sialic acid polymer, as a modifier and reported that polysialylation of enzymes improves the stability and increases the half-life in the blood circulation [63]. Maeda and his group conjugated poly(styrene-co-maleic acid) butyl ester (SMA) to SOD [64] and found that intravenously administered SMA–SOD bound to albumin and circulated with an extremely long half-life of 6 h, whereas unmodified SOD rapidly underwent renal glomerular filtration with a plasma half-life of only 4 min.

In general, cellular uptake is also greatly reduced by pegylation. Although uricase and bovine liver catalase disappeared rapidly from the plasma due to uptake by organs such as the liver after intravenous injection into mice, the plasma half-life of these proteins was significantly increased by pegylation [40]. Yabe *et al.* reported that pegylation increased the AUC by ~ 70-fold, and reduced the apparent hepatic uptake clearance of catalase to < 1% [40]. The uptake of cationic proteins by organs is also slowed down by pegylation, and so the rate of delivery can be precisely regulated when pegylation and other chemical modification techniques are used simultaneously.

A variety of reagents for pegylation, which have at least one functional group that is capable of conjugation with proteins, are commercially available [61]. In most cases, amino groups or carboxyl groups are used for conjugation. When reagents that form an ester bond are used, the PEG chain may be detached from the conjugated proteins during the circulation; and this is an effective method for precisely controlling the plasma half-life of proteins.

Enhanced retention of therapeutic proteins in the circulation would be advantageous for their passive targeting to tissues and organs where the permeability of capillaries is high enough to allow the passage of proteins [44]. High vascular permeability and a sparse interstitial structure of solid tumour tissues seem to facilitate the migration of circulating macromolecules to tumour tissues. In addition, a lack of functional lymphatic drainage will result in the accumulation of extravasated solutes by a passive mechanism. These characteristics of tumour tissues are referred to as the enhanced permeability and retention effect [65], which provides a reliable rationale for

the use of macromolecules and particulates with a long plasma half-life for tumour targeting.

3.3 Increased binding to the cell surface by cationisation

Cell surface membranes are negatively charged due to the presence of large amounts of proteoglycans and other anionic molecules. This charged surface provides the site of interaction for cationic compounds. Therefore, increasing the electric charge of proteins, which is called cationisation, would increase the binding of proteins to various types of cells through electrostatic interaction. In general, cationic compounds are more extensively bound to cells than their neutral or anionic counterparts [66,67]. Because of the simple basis of the interaction, cationisation is a universal approach for increasing the cellular binding and uptake of many different macromolecules, and it is used for the targeted delivery of drugs, proteins and genes [68]. Diamines, such as ethylenediamine and hexamethylenediamine, are frequently used for the preparation of cationised proteins [69].

Because of their strong interaction with different cells, positively charged compounds have a shorter circulation time in the plasma than their neutral and/or negatively charged counterparts after intravenous injection. The liver plays a significant role in the overall clearance of cationic macromolecules from the circulation because of its anatomical and physiological properties (i.e., the discontinuous endothelium, the high endocytic activity of the liver cells and the large surface area). The kidneys are also important organs for the uptake of cationic macromolecules. Some cationic compounds, such as the cationic particles that are composed of plasmid DNA and cationic liposomes, are trapped by the lung after systemic administration, when the interaction of such complexes with serum proteins and/or blood cells is involved.

Pardridge and colleagues published a series of papers in which they reported that cationised serum albumin, IgG and other proteins can strongly bind to microvascular endothelial cells in the brain [66]. Ma *et al.* reported that chemically cationised bovine serum albumin (BSA) derivatives are selectively delivered to the liver, depending on the degree of cationisation [69]. Following intravenous administration, cationised SOD was delivered to the kidneys, and exhibited a more significant effect on ischaemic acute renal failure than unmodified SOD [70]. Cationised catalase and horseradish peroxidase markedly suppressed antigen-induced arthritis in a zymosan-induced model of arthritis when injected into mouse knee joints [71]. The half-life of these enzymes in the joint was significantly extended compared with that of native enzymes.

3.4 Targeted delivery by glycosylation

Various combinations of ligands and receptors have been investigated as a means of achieving target-specific delivery of pharmaceuticals. However, it is important for any ligand to have access to the target cells that carry the receptors in order for the ligand to be recognised by its corresponding receptors.

The sugar–lectin interaction has several features that are appropriate for the cell-specific targeting of pharmaceuticals:

- the expression of lectins is specific to some types of cells
- the affinity of the ligands can be high enough to achieve efficient *in vivo* targeting
- the use of the recognition system produces little interference with the interactions that are important for life

The most frequently used receptors for targeted drug delivery are the asialoglycoprotein receptors on hepatocytes, which recognise galactose and *N*-acetylgalactosamine on the nonreducing terminal of sugar chains. Morell *et al.* carried out pioneering work to show that glycoproteins are rapidly cleared from the plasma circulation by the liver once the sialic acids on the nonreducing terminal of the sugar chains are removed [72]. Because other normal cells express hardly any receptors that recognise galactose, the uptake of galactose-exposing compounds is highly specific to hepatocytes. Therefore, this recognition has been applied to the receptor-mediated targeting of pharmaceuticals since 1971, using not only asialoglycoproteins but also chemically galactosylated proteins [73].

The interaction of galactosylated ligands with asialoglycoprotein receptors has been extensively investigated using isolated hepatocytes. Connolly *et al.* reported that highly clustered galactosides were more potent inhibitors than less clustered ones in their studies of the binding of asialoorosomucoid to hepatocytes [74]. Nishikawa and colleagues have developed galactosylated proteins that have different molecular weights and different numbers of galactose units, and investigated their tissue distribution in mice and rats after intravenous injection [48,75,76]. Proteins such as BSA, mouse IgG, human Cu^{2+}, Zn^{2+}-SOD, bovine liver catalase, soybean trypsin inhibitor and chicken egg lysozyme were successfully delivered to the liver by galactosylation. These galactosylated proteins were significantly delivered to hepatocytes, cells that possess asialoglycoprotein receptors, when administered to the systemic circulation. Although all galactosylated proteins were delivered to the liver, the rate and extent of this delivery was highly dependent on the molecular weight of the proteins, the number of galactose residues and the administered dose. A pharmacokinetic analysis based on a physiological model showed that the surface density of the galactose residues on proteins correlated well with the affinity of the galactosylated proteins for the receptors [76]. These results indicate that an efficient delivery of proteins to hepatocytes by galactosylation can be achieved by adjusting the degree of galactosylation (e.g., by ensuring that the distance between two vicinal galactose residues is as short as 20 – 30 Å), which is of the same order as the naturally occurring sugar clusters that are arranged at the vertices of a triangle with sides of dimensions 15, 22 and 25 Å. Mannosylation is another glycosylation technique that can be used for the targeted delivery of proteins to cells that express mannose receptors. Although mannose receptors are expressed on macrophages and other cells, mannosylated proteins injected intravenously are selectively

delivered to liver nonparenchymal cells such as sinusoidal endothelial cells and Kupffer cells [75,77]. Again, various proteins and other macromolecules can be targeted to mannose receptor-positive cells by mannosylation [40,78,79]. Mannan-binding protein, another mannose-recognising lectin that circulates in the blood, affects the tissue distribution of mannosylated ligands, as observed with BSA derivatives that have been extensively modified with mannose [77].

Although the ligand–receptor interaction is the driving force for the targeted delivery of glycosylated proteins to liver cells, additional factors such as blood flow rate, capillary structure and interaction with blood components [68] will affect the overall interaction with receptors *in vivo*. Rapid elimination by glomerular filtration may be another factor hindering efficient delivery. Increasing the administered dose markedly reduces the delivery efficiency to the liver when the molecular size of the protein is smaller than the glomerular filtration threshold [76]. During the modification procedures, care should be taken not to alter the electric charge of the proteins, because a reduction in the charge sometimes increases the affinity of the protein for scavenger receptors [80] as described in Section 3.5.

3.5 Targeted delivery via scavenger receptor-mediated uptake

Although many proteins are negatively charged at a physiological pH, a further reduction in the net electric charge by chemical modification will significantly alter their tissue distribution. Yamasaki *et al.* [51] showed that BSA derivatives modified with succinic anhydride are cleared by the liver after intravenous injection at a rate that depends on the degree of modification. This can be explained by the fact that scavenger receptors that recognise compounds with a strong negative charge are expressed on sinusoidal endothelial cells and Kupffer cells [81]. Therefore, chemical modification techniques to reduce the net charge of proteins are used to prepare derivatives that efficiently target the liver nonparenchymal cells. In addition to succinic anhydride, maleic anhydride and aconitic acid are reagents that are frequently used for the preparation of ligands for the receptors. When some ligands are coupled to the amino groups on proteins, such protein derivatives may be recognised by the scavenger receptors, even though no such recognition is intended, as demonstrated in a study using glycosylated proteins [80].

3.6 Conjugation with monoclonal antibody

Apart from ligands that are recognised by receptors, mAbs, first described by Kohler and Milstein in 1975 [82], can be used for the targeted delivery of pharmaceutical compounds to cells that have the corresponding antigens on the cell surface. In recent years, recombinant antibody engineering techniques have been used to construct novel and potentially effective molecules. Techniques to produce human-type mAbs have been developed and the products obtained have a reduced immunogenicity. A mAb to platelet-endothelial cell adhesion molecule-1 was used for targeting catalase to the endothelial cells in the lung, which this was found to be effective in reducing both oxidative stress and acute lung-graft injury after prolonged cold storage [83].

4. Inhibition of tumour metastasis by controlled delivery of catalase

Although it is accepted that ROS are involved in the metastatic processes of tumour cells, a few investigators have tried to inhibit tumour metastasis by using antioxidant enzymes or other antioxidants. In addition, the results that were obtained with SODs and catalase, the two major groups of antioxidant enzymes used in those studies, vary from laboratory to laboratory, depending on the enzymes, tumour cells and experimental systems used [33,84-88].

SODs and catalase are sometimes classified together as antioxidant enzymes but they should be treated separately because their functions are completely different. All types of SODs accelerate the dismutation rate of superoxide anion and produce hydrogen peroxide and oxygen. On the other hand, catalase catabolises hydrogen peroxide to water and oxygen. Therefore, SODs increase the concentration of hydrogen peroxide, whereas catalase reduces it. As discussed in Section 2.2, hydrogen peroxide is a very important molecule in regulating the transcription of various genes. Therefore, it was considered that the elimination of hydrogen peroxide at the site where tumour metastasis occurs could inhibit or prevent tumour metastasis.

4.1 Targeted delivery to hepatocytes

The liver is a common site of metastasis of colorectal cancer cells. As discussed in Sections 3.3 – 3.5, targeted delivery of catalase to the liver can be achieved by several types of chemical modification [40]. Galactosylated catalase is selectively delivered to hepatocytes (liver parenchymal cells), whereas mannosylated, fusocylated or succinylated catalase is preferentially delivered to liver nonparenchymal cells such as Kupffer cells and sinusoidal endothelial cells. Chemically modified catalase derivatives showed significantly higher therapeutic activities than their unmodified counterpart in preventing hepatic ischaemia/reperfusion injuries [40,79]; succinylated catalase markedly suppressed the increase in the expression of intercellular adhesion molecule-1 along the hepatic sinusoid (Figure 4) and prevented neutrophil infiltration in the liver [79].

Nishikawa and colleagues examined the effects of catalase derivatives with different tissue distribution properties on hepatic metastasis in mice, by injecting 1×10^5 mouse colon carcinoma colon 26 cells into the portal vein [89]. Metastatic colonies in the liver were clearly visible at 2 weeks after inoculation and > 50 colonies were detected on the surface of this organ. In accordance with the formation of many metastatic colonies, the weight of the liver of tumour-bearing mice was significantly greater than that of control mice. These results indicate that some colon 26 cells inoculated via the portal vein

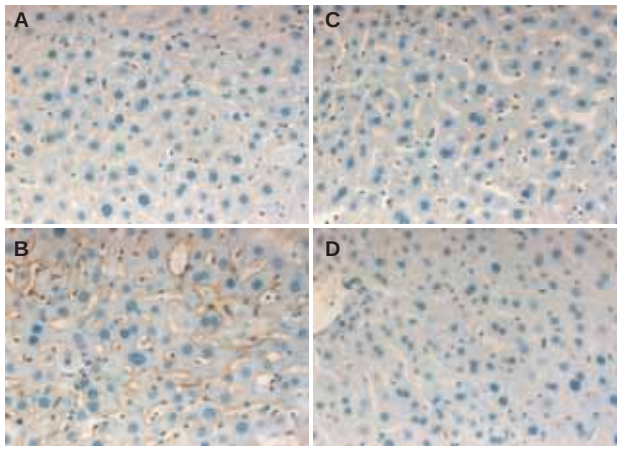

Figure 4. Expression of ICAM-1 in liver sections of mice at 30 min after a period of ischaemia followed by 30 min of reperfusion. Under anaesthesia, the portal vein and hepatic artery of mice were occluded with a vascular clamp for 30 min to induce hepatic ischaemia. Blood was then allowed to flow through the liver again (reperfusion). Saline (control), catalase (10,000 U/kg) or a combination of succinylated catalase and mannosylated SOD (10,000 U/kg for each) was administered twice through the tail vein 5 min before and 60 min after re-establishing blood flow. After 30 min of reperfusion, liver tissues were excised and fixed in 10% neutral, buffered formalin, embedded in paraffin blocks and 5-μm thick sections were prepared. The sections were then stained with a rat antimouse ICAM-1 antibody. **A)** Control (normal liver); **B)** saline; **C)** catalase; **D)** succinylated catalase and mannosylated SOD. Reprinted from YABE Y, KOBAYASHI N, NISHIHASHI T *et al.*: Prevention of neutrophil-mediated hepatic ischaemia/reperfusion injury by superoxide dismutase and catalase derivatives. *J. Pharmacol. Exp. Ther.* (2001) **298**(3):894-899 [79]. Copyright (2001), with permission from the American Society for Pharmacology and Experimental Therapeutics.
ICAM: Intercellular adhesion molecule; SOD: Superoxide dismutase.

survive, adhere to the endothelial cells, invade the tissue parenchyma and then proliferate to form metastatic colonies. Because the colon 26 cells that were injected into the tail vein form metastatic colonies only in the lung (see Section 4.2), the initial step in the formation of metastasis of the colon 26 cells would be mediated by the physical trapping of the cells within the microvasculature of the organs rather than by a specific interaction between the tumour and endothelial cells.

An intravenous injection of any catalase derivative (i.e., unmodified, galactosylated, mannosylated and succinylated catalase) significantly reduces the number of metastatic colonies in the liver. However, of those examined, galactosylated catalase (a derivative targeting hepatocytes via an asialoglycoprotein receptor-mediated process) produced the highest reduction in the number of metastatic colonies. The number of colonies was significantly ($p < 0.001$) smaller than that obtained under any of the other conditions examined. In the same model, neither BSA nor inactivated catalase had a significant effect on the number of metastatic colonies. Thus, this strongly suggests that the inhibitory effects of catalase derivatives on tumour metastasis are due to the action of the catalase in detoxifying hydrogen peroxide. In addition, the importance of the targeted delivery of catalase activity to hepatocytes is clearly demonstrated by the inhibition of hepatic metastasis of colorectal cancer cells.

Hepatic sinusoidal cells (i.e., Kupffer cells and endothelial cells) play an important role in the early phase of hepatic metastasis. Early studies demonstrated that the inhibition of Kupffer cell functions prior to tumour-cell challenge increases metastatic growth in the liver [90], indicating that Kupffer cells act as a defence against hepatic metastasis. They can arrest circulating tumour cells and kill them either directly or by recruiting inflammatory cells. Therefore, activation of Kupffer cells is one method of inhibiting hepatic metastasis, although their ability to kill tumour cells is somewhat limited to a small number of tumour cells. Ischaemia/reperfusion injury, which will occur following the entry and subsequent removal of tumour cells in the hepatic microcirculation, is involved in this defence mechanism against hepatic metastasis by sinusoidal cells. In addition to these sinusoidal cells, natural killer cells have also been found to be effective in preventing hepatic metastasis [91]. Thus, these cells build a defence system to block tumour metastasis, but they exhibit little tumoricidal activity at several days after the first encounter with tumour cells [90].

The main proteolytic enzymes that are involved in tumour metastasis are MMPs. In Nishikawa's study using the hepatic metastasis model of colon 26 cells [89], a significant amount of gelatinase activity was detected in liver containing metastatic colonies of colon 26 cells. Delivery of catalase to hepatocytes by galactosylation significantly reduced the total MMP activity in the liver with metastatic colonies. A gelatin zymographic analysis of the liver homogenates indicated that MMP-9 activity was increased by the inoculation of tumour cells but this largely (> 90%) disappeared following the administration of galactosylated catalase. Previous studies have indicated that cellular MMP activities are, at least partially, regulated by the concentration of hydrogen peroxide [29]. Wenk *et al.* showed that hydrogen peroxide increases MMP-1 mRNA levels by using Mn–SOD-overexpressing fibroblasts [92]. In addition, Nelson *et al.* reported that elevated SOD activity increases MMP expression by increasing the hydrogen peroxide level in tumour cells [33]. Therefore, the suppression of MMP activity by detoxifying hydrogen peroxide would explain the inhibitory effect of galactosylated catalase. *In situ* zymography suggested that the gelatinase activity in the tumour-bearing liver is close to the hepatic sinusoids. MMPs are known to be produced from various cells, including tumour cells, endothelial cells, macrophages and hepatocytes [28,29]. The precise mechanism for the increase in MMP activity and its prevention by galactosylated catalase needs to be investigated. However, it is suggested that the increase in MMP activity released from hepatocytes is inhibited by targeted delivery of catalase to the cells, and this

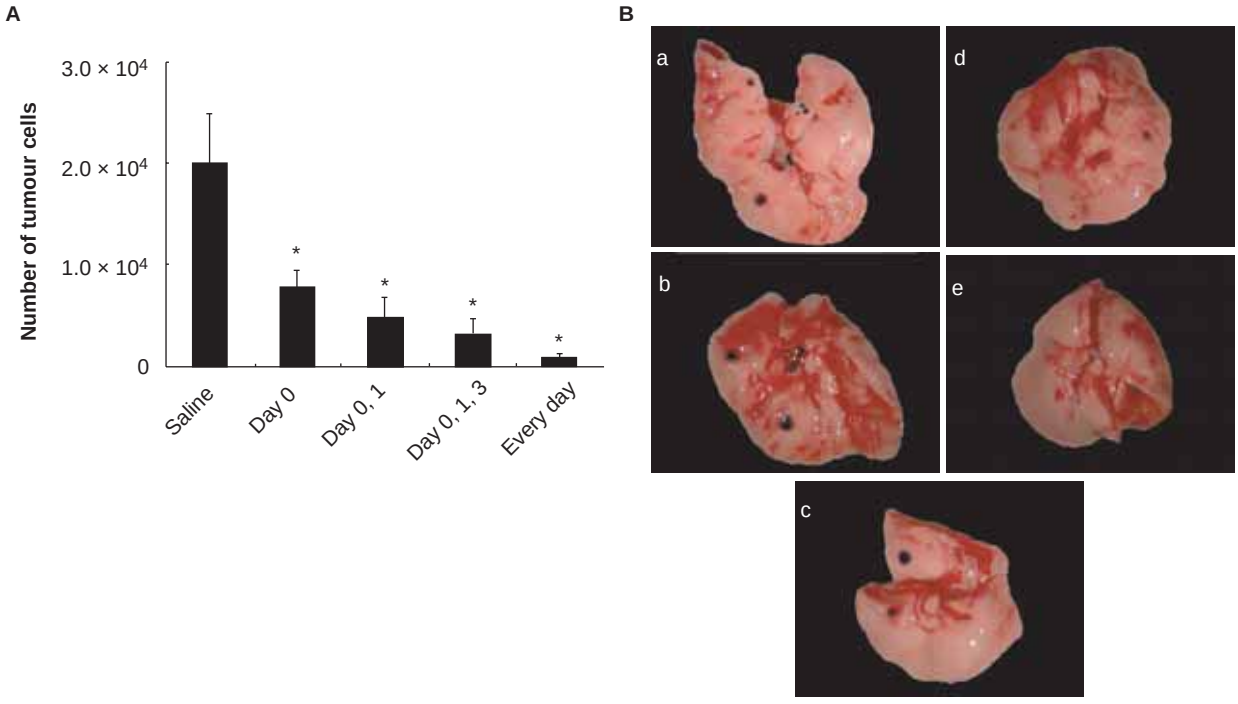

Figure 5. Effect of PEG–catalase on pulmonary metastasis in mice. Experimental pulmonary metastasis was induced by injecting 1×10^4 B16-BL6 cells that were stably expressing firefly luciferase gene (B16-BL6/Luc cells) into the tail vein of C57-BL6 mice. Saline (untreated, control group) or PEG–catalase (1000 U/injection) was intravenously injected into mice according to the schedule indicated. **A)** Mice were killed at 14 days after tumour injection and the luciferase activity in the lung was assayed. Results are expressed as the mean ± standard deviation of at least six mice. **B)** Typical examples of pulmonary metastases in mice receiving an intravenous injection of 1×10^4 B16-BL6/Luc cells followed by an injection of PEG–catalase. a) Saline (vehicle), b) PEG–catalase (day 0), c) PEG–catalase (day 0, 1), d) PEG–catalase (day 0, 1, 3), e) PEG–catalase (every day). Each catalase derivative was injected into the tail vein at a dose of 1000 U/injection. Reprinted from HYOUDOU K, NISHIKAWA M, UMEYAMA Y, KOBAYASHI Y, YAMASHITA F, HASHIDA M: Inhibition of metastatic tumour growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells. *Clin.Cancer Res.* (2004) **10**(22):7685-7691 [18]. Copyright (2004), with permission from the American Association for Cancer Research.

*A statistically significant difference compared with the saline group ($p < 0.001$).
PEG: Poly(ethylene glycol).

inhibition prevents the development of hepatic metastases of colon carcinoma cells.

4.2 Prolonged circulation

In many cases, the lungs are the first organs to be encountered by tumour cells that are detached from primary tumours and this makes it a major site for tumour metastasis. Pulmonary delivery of catalase may be achieved by conjugation with antiplatelet-endothelial cell adhesion molecule-1 antibody [83] or by the use of cationic liposomes [68]. As an alternative, Nishikawa and colleagues have been trying to inhibit pulmonary metastasis by increasing the plasma half-life of catalase by pegylation [18,93].

Although PEG–catalase did not exhibit pronounced accumulation in the lungs, it had a long plasma half-life and produced ~ 70-fold greater AUC than unmodified catalase [40,93]. In an experimental pulmonary metastasis model of colon 26 cells in mice, a single injection of PEG–catalase significantly reduced the number of metastatic colonies on the lung surface [93]. After injection of 1×10^5 colon 26 cells into the tail vein, the number of metastatic colonies in the lung was 93 ± 29 for the saline (vehicle) treatment group at 2 weeks. An intravenous injection of catalase at a dose of 35,000 U/kg significantly ($p < 0.01$) reduced the number of metastatic colonies to 63 ± 23. PEG-catalase at the same dose produced an inhibitory effect on the number of colonies in the lung greater than other catalase derivatives, and only 22 ± 11 colonies were found ($p < 0.001$ compared with the saline treatment group, and $p < 0.01$ compared with the catalase treatment group). Again, neither inactivated catalase nor BSA had any effect on the number of metastases. The catalase activity in the lung of mice with pulmonary colonies was significantly lower than that of control mice and increasing the tissue weight due to the growth of the tumour colonies reduced the catalase activity. Therefore, it is suggested that tumour tissues exhibit less catalase activity than normal lung tissue.

The processes of pulmonary metastasis of tumour cells and the following proliferation in the organ were then quantitatively

examined using mouse melanoma B16-BL6 cells stably (permanently) labelled with firefly luciferase (B16-BL6/Luc cells) [18]. Tumour cells labelled with firefly luciferase, a green fluorescent protein, or other proteins have been used to study tumour metastasis [94-96]. These cells are very useful for evaluating tumour metastasis because the protein introduced is highly tumour-cell specific. In the study by Hyoudou *et al.*, B16-BL6/Luc cells in the lung could be detected by measuring the luciferase activity with a very low detection limit of 60 tumour cells in the organ [18]. B16-BL6/Luc cells were mostly (60 – 90%) trapped by the lung just after inoculation into the tail vein, and the number was reduced to ~ 2 – 4% by 24 h. The number then increased with time to values exceeding the numbers injected, indicating that the surviving tumour cells started to proliferate in the lungs. Thus, sequential measurements of the luciferase activity in the lungs can provide an overview of the events occurring during the metastasis of tumour cells. After injection into the tail vein of mice, B16-BL6/Luc cells are trapped by the microvasculature in the lungs and most of them are destroyed within the first 24 h, although the few surviving would extravasate. From day 1 – 3, the tumour cells could invade the tissue parenchyma and start to proliferate.

Administration of PEG–catalase just before tumour inoculation reduced the number of B16-BL6/Luc cells in the lung 24 h after tumour inoculation, suggesting that PEG–catalase inhibits the adhesion of tumour cells to the endothelium or other very early steps of metastasis. In addition, PEG–catalase was also effective in reducing the number of tumour cells at day 7, when injected intravenously 1 or 3 days after tumour inoculation. Because the tumour cells in the lungs are in growth phase 3 days after tumour inoculation, these results indicate that scavenging of hydrogen peroxide by PEG–catalase can also inhibit the proliferation of metastatic tumour cells. Thus, it seems that PEG–catalase inhibits not only the adhesion of tumour cells but also their invasion as well as proliferation. Daily dosing of PEG–catalase greatly inhibited the proliferation of the tumour cells that were present in the lungs (Figure 5). The daily injection of PEG–catalase ≤ 30 days after tumour inoculation significantly prolonged the survival rate of mice with B16-BL6 lung metastases compared with the saline- or BSA-treatment groups. These findings indicate that sustained catalase activity in the blood circulation can prevent the multiple processes of tumour metastasis, including adhesion and proliferation, which may lead to a state of tumour dormancy.

5. Expert opinion and conclusion

Studying the antimetastatic properties of any pharmaceutical agent is a difficult task because most experimental models of tumour metastasis require administration or inoculation of tumour cells into blood vessels or cavities. This is a completely different situation from cancer patients, who never receive injections of cancer cells. Therefore, tumour metastasis may be more properly evaluated in spontaneous models, in which tumour cells metastasise to sites in the body that are different from that where they are inoculated. Such models, however, require a much longer time for investigation than experimental models and exhibit large deviations in results. Thus, developing a proper spontaneous metastatic model is of great importance in establishing an effective therapeutic agent for inhibiting tumour metastasis. It is important to note that metastatic tumour cells sometimes remain dormant in tissues to which they have migrated, but they can then start to proliferate after a long interval following dissociation from primary tumours. These clinical and experimental observations [97,98] confirm that migrating tumour cells do not indiscriminately produce secondary tumours wherever they become located.

Recent studies have suggested the possibility of tumour dormancy; solitary cancer cells exhibit very little proliferation or apoptosis [99,100]. If these quiescent cells remain viable in sufficiently large numbers, they could contribute to metastatic recurrence after a period of clinical dormancy. Although surgical removal is a primary option for patients with removable solid tumours, a correlation between surgical trauma and locoregional tumour recurrence has been demonstrated [101,102]. It is also reported that surgical trauma will result in the production of ROS from inflammatory cells entering damaged tissues via a respiratory burst. Thus, catalase derivatives may also be effective against metastatic tumour growth after surgical removal of primary tumour tissues. Nishikawa and colleagues are now trying to prove this using a spontaneous metastatic model of B16-BL6/Luc cells inoculated into the mouse footpad. In recent years, hydrogen peroxide has been found to activate cellular proliferation and act as an endothelium-derived hyperpolarising factor [103]. Therefore, attention should be paid to how detoxifying hydrogen peroxide interferes with its physiological functions in the development of catalase derivatives, with optimised tissue distribution properties for the inhibition of tumour metastasis.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. ENGERS R, GABBERT HE: Mechanisms of tumor metastasis: cell biological aspects and clinical implications. *J. Cancer Res. Clin. Oncol.* (2000) 126(12):682-692.
2. NISHIKAWA M, HYOUDOU K, KOBAYASHI Y, UMEYAMA Y, TAKAKURA Y, HASHIDA M: Inhibition of metastatic tumour growth by targeted delivery of antioxidant enzymes. *J. Control. Release* (2005) 109(1-3):101-107.
3. ZETTER BR: The cellular basis of site-specific tumor metastasis. *N. Engl. J. Med.* (1990) 322(9):605-612.
4. TANG DG, HONN KV: Adhesion molecules and tumor metastasis: an update. *Invasion Metastasis* (1994-1995) 14(1-6):109-122.
5. LI C, JACKSON RM: Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am. J. Physiol.* (2002) 282(2):C227-C241.

6. BARBERA-GUILLEM E, WEISS L: Cancer-cell traffic in the liver. III. Lethal deformation of B16 melanoma cells in liver sinusoids. *Int. J. Cancer* (1993) 54(5):880-884.
7. JESSUP JM, BATTLE P, WALLER H *et al.*: Reactive nitrogen and oxygen radicals formed during hepatic ischemia-reperfusion kill weakly metastatic colorectal cancer cells. *Cancer Res.* (1999) 59(8):1825-1829.
8. WANG HH, MCINTOSH AR, HASINOFF BB *et al.*: B16 melanoma cell arrest in the mouse liver induces nitric oxide release and sinusoidal cytotoxicity: a natural hepatic defense against metastasis. *Cancer Res.* (2000) 60(20):5862-5869.
9. LAURENT A, NICCO C, CHEREAU C *et al.*: Controlling tumor growth by modulating endogenous production of reactive oxygen species. *Cancer Res.* (2005) 65(3):948-956.
10. SZATROWSKI TP, NATHAN CF: Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* (1991) 51(3):794-798.
11. TOYOKUNI S: Oxidative stress and cancer: the role of redox regulation. *Biotherapy* (1998) 11(2-3):147-154.
12. BEHREND L, HENDERSON G, ZWACKA RM: Reactive oxygen species in oncogenic transformation. *Biochem. Soc. Trans.* (2003) 31(6):1441-1444.
13. CROSS AR, JONES OT: Enzymic mechanisms of superoxide production. *Biochim. Biophys. Acta* (1991) 1057(3):281-298.
14. BURDON RH: Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic. Biol. Med.* (1995) 18(4):775-794.
- A thorough review on the involvement of ROS in the proliferation of mammalian cells.
15. MYTAR B, SIEDLAR M, WOLOSZYN M, RUGGIERO I, PRYJMA J, ZEMBALA M: Induction of reactive oxygen intermediates in human monocytes by tumour cells and their role in spontaneous monocyte cytotoxicity. *Br. J. Cancer* (1999) 79(5-6):737-743.
16. MEIER B, RADEKE HH, SELLE S *et al.*: Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-α. *Biochem. J.* (1989) 263(2):539-545.
17. SCHRAUFSTATTER IU, HYSLOP PA, HINSHAW DB, SPRAGG RG, SKLAR LA, COCHRANE CG: Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA* (1986) 83(13):4908-4912.
18. HYOUDOU K, NISHIKAWA M, UMEYAMA Y, KOBAYASHI Y, YAMASHITA F, HASHIDA M: Inhibition of metastatic tumor growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells. *Clin. Cancer. Res.* (2004) 10(22):7685-7691.
19. PELICANO H, CARNEY D, HUANG P: ROS stress in cancer cells and therapeutic implications. *Drug. Resist. Updat.* (2004) 7(2):97-110.
- Suggests therapeutic strategies that take advantage of increased ROS in cancer cells to enhance therapeutic activity.
20. CHAN WH, YU JS: Inhibition of UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermal carcinoma A431 cells by genistein. *J. Cell. Biochem.* (2000) 78(1):73-84.
21. SERRANO J, PALMEIRA CM, KUEHL DW, WALLACE KB: Cardioselective and cumulative oxidation of mitochondrial DNA following subchronic doxorubicin administration. *Biochim. Biophys. Acta* (1999) 1411(1):201-205.
22. HUG H, STRAND S, GRAMBIHLER A *et al.*: Reactive oxygen intermediates are involved in the induction of CD95 ligand mRNA expression by cytostatic drugs in hepatoma cells. *J. Biol. Chem.* (1997) 272(45):28191-28193.
23. MIYAJIMA A, NAKASHIMA J, YOSHIOKA K, TACHIBANA M, TAZAKI H, MURAI M: Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. *Br. J. Cancer* (1997) 76(2):206-210.
24. SAWA T, WU J, AKAIKE T, MAEDA H: Tumor-targeting chemotherapy by a xanthine oxidase-polymer conjugate that generates oxygen-free radicals in tumor tissue. *Cancer Res.* (2000) 60(3):666-671.
- Shows the first experimental evidence that the delivery of a ROS-generating enzyme to tumour tissue is effective in killing tumour cells.
25. SEN CK, PACKER L: Antioxidant and redox regulation of gene transcription. *FASEB J.* (1996) 10(7):709-720.
26. SUN Y, OBERLEY LW: Redox regulation of transcriptional activators. *Free Radic. Biol. Med.* (1996) 21(3):335-348.
27. ALLEN RG, TRESINI M: Oxidative stress and gene regulation. *Free Radic. Biol. Med.* (2000) 28(3):463-499.
- An excellent review on the ROS-mediated transcriptional regulation of various genes.
28. SHAUGHNESSY SG, WHALEY M, LAFRENIE RM, ORR FW: Walker 256 tumor cell degradation of extracellular matrices involves a latent gelatinase activated by reactive oxygen species. *Arch. Biochem. Biophys.* (1993) 304(2):314-321.
29. BELKHIRI A, RICHARDS C, WHALEY M, MCQUEEN SA, ORR FW: Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H_2O_2 exposure. *Lab. Invest.* (1997) 77(5):533-539.
30. SELLAK H, FRANZINI E, HAKIM J, PASQUIER C: Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood* (1994) 83(9):2669-2677.
31. KNEBEL A, RAHMSDORF HJ, ULLRICH A, HERRLICH P: Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *EMBO J.* (1996) 15(19):5314-5325.
32. CHUA CC, HAMDY RC, CHUA BH: Upregulation of vascular endothelial growth factor by H_2O_2 in rat heart endothelial cells. *Free Radic. Biol. Med.* (1998) 25(8):891-897.
33. NELSON KK, RANGANATHAN AC, MANSOURI J *et al.*: Elevated *Sod2* activity augments matrix metalloproteinase expression: evidence for the involvement of endogenous hydrogen peroxide in regulating metastasis. *Clin. Cancer Res.* (2003) 9(1):424-432.
34. CHENG GC, SCHULZE PC, LEE RT, SYLVAN J, ZETTER BR, HUANG H: Oxidative stress and thioredoxin-interacting protein promote intravasation of melanoma cells. *Exp. Cell Res.* (2004) 300(2):297-307.
35. SUNDARESAN M, YU ZX, FERRANS VJ, IRANI K, FINKEL T: Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* (1995) 270(5234):296-299.

36. ARNOLD RS, SHI J, MURAD E *et al.*: Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc. Natl. Acad. Sci. USA* (2001) 98(10):5550-5555.

37. YABE Y, KOYAMA Y, NISHIKAWA M, TAKAKURA Y, HASHIDA M: Hepatocyte-specific distribution of catalase and its inhibitory effect on hepatic ischemia/reperfusion injury in mice. *Free Radic. Res.* (1999) 30(4):265-274.

38. TAKAKURA Y, FUJITA T, HASHIDA M, SEZAKI H: Disposition characteristics of macromolecules in tumor-bearing mice. *Pharm. Res.* (1990) 7(4):339-346.

39. SEYMOUR LW, MIYAMOTO Y, MAEDA H *et al.*: Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *Eur. J. Cancer* (1995) 31A(5):766-770.

40. YABE Y, NISHIKAWA M, TAMADA A, TAKAKURA Y, HASHIDA M: Targeted delivery and improved therapeutic potential of catalase by chemical modification: combination with superoxide dismutase derivatives. *J. Pharmacol. Exp. Ther.* (1999) 289(2):1176-1184.

41. NARA E, MASEGI M, HATONO T, HASHIDA M: Pharmacokinetic analysis of drug absorption from muscle based on a physiological diffusion model: effect of molecular size on absorption. *Pharm. Res.* (1992) 9(2):161-168.

42. NOMURA T, NAKAJIMA S, KAWABATA K, YAMASHITA F, TAKAKURA Y, HASHIDA M: Intratumoral pharmacokinetics and *in vivo* gene expression of naked plasmid DNA and its cationic liposome complexes after direct gene transfer. *Cancer Res.* (1997) 57(13):2681-2686.

43. NOMURA T, SAIKAWA A, MORITA S *et al.*: Pharmacokinetic characteristics and therapeutic effects of mitomycin C-dextran conjugates after intratumoural injection. *J. Control. Release* (1998) 52(3):239-252.

44. SEZAKI H, HASHIDA M: Macromolecule-drug conjugates in targeted cancer chemotherapy. *Crit. Rev. Ther. Drug Carrier Syst.* (1984) 1(1):1-38.

45. SIMIONESCU N: Cellular aspects of transcapillary exchange. *Physiol. Rev.* (1983) 63(4):1536-1579.

46. MATSUMURA Y, MAEDA H: A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res.* (1986) 46(12 Pt 1):6387-6392.

• Proves that macromolecules accumulate in solid tumours due to an enhanced permeability and retention effect.

47. BRENNER BM, HOSTETTER TH, HUMES HD: Glomerular permselectivity: barrier function based on discrimination of molecular size and charge. *Am. J. Physiol.* (1978) 234(6):F455-F460.

48. NISHIKAWA M, HIRABAYASHI H, TAKAKURA Y, HASHIDA M: Design for cell-specific targeting of proteins utilizing sugar-recognition mechanism: effect of molecular weight of proteins on targeting efficiency. *Pharm. Res.* (1995) 12(2):209-214.

49. MIHARA K, HOJO T, FUJIKAWA M, TAKAKURA Y, SEZAKI H, HASHIDA M: Disposition characteristics of protein drugs in the perfused rat kidney. *Pharm. Res.* (1993) 10(6):823-827.

50. MIYAZAKI A, NAKAYAMA H, HORIUCHI S: Scavenger receptors that recognize advanced glycation end products. *Trends Cardiovasc. Med.* (2002) 12(6):258-262.

51. YAMASAKI Y, SUMIMOTO K, NISHIKAWA M *et al.*: Pharmacokinetic analysis of *in vivo* disposition of succinylated proteins targeted to liver nonparenchymal cells via scavenger receptors: importance of molecular size and negative charge density for *in vivo* recognition by receptors. *J. Pharmacol. Exp. Ther.* (2002) 301(2):467-477.

52. TAKAKURA Y, MAHATO RI, HASHIDA M: Extravasation of macromolecules. *Adv. Drug Deliv. Rev.* (1998) 34(1):93-108.

53. RAPOPORT SI: Osmotic opening of the blood-brain barrier: principles, mechanism, and therapeutic applications. *Cell. Mol. Neurobiol.* (2000) 20(2):217-230.

54. LIU F, SONG Y, LIU D: Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* (1999) 6(7):1258-1266.

55. KOBAYASHI N, NISHIKAWA M, TAKAKURA Y: The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. *Adv. Drug Deliv. Rev.* (2005) 57(5):713-731.

56. KOBAYASHI N, NISHIKAWA M, HIRATA K, TAKAKURA Y: Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. *J. Gene Med.* (2004) 6(5):584-592.

57. ZHANG G, GAO X, SONG YK *et al.*: Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* (2004) 11(8):675-682.

58. LIANG KW, NISHIKAWA M, LIU F, SUN B, YE Q, HUANG L: Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA. *Gene Ther.* (2004) 11(11):901-908.

59. SEZAKI H, TAKAKURA Y, HASHIDA M: Soluble macromolecular carriers for the delivery of antitumor drugs. *Adv. Drug Deliv. Rev.* (1989) 3:247-266.

60. NISHIKAWA M, TAKAKURA Y, HASHIDA M: Theoretical considerations involving the pharmacokinetics of plasmid DNA. *Adv. Drug Deliv. Rev.* (2005) 57(5):675-688.

61. ROBERTS MJ, BENTLEY MD, HARRIS JM: Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* (2002) 54(4):459-476.

•• An excellent review on the chemistry and applications of pegylation for peptides and proteins.

62. VERONESE FM, CALICETI P, SCHIAVON O, SERGI M: Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv. Drug Deliv. Rev.* (2002) 54(4):587-606.

63. GREGORIADIS G, MCCORMACK B, WANG Z, LIFELY R: Polysialic acids: potential in drug delivery. *FEBS Lett.* (1993) 315(3):271-276.

64. OGINO T, INOUE M, ANDO Y, AWAI M, MAEDA H, MORINO Y: Chemical modification of superoxide dismutase. Extension of plasma half life of the enzyme through its reversible binding to the circulating albumin. *Int. J. Pept. Protein Res.* (1988) 32(2):153-159.

65. MAEDA H, WU J, SAWA T, MATSUMURA Y, HORI K: Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control. Release* (2000) 65(1-2):271-284.

66. TRIGUERO D, BUCIAK JB, YANG J, PARDRIDGE WM: Blood-brain barrier

transport of cationized immunoglobulin G: enhanced delivery compared to native protein. *Proc. Natl. Acad. Sci. USA* (1989) 86(12):4761-4765.

67. NISHIKAWA M, HASEGAWA S, YAMASHITA F, TAKAKURA Y, HASHIDA M: Electrical charge on protein regulates its absorption from the rat small intestine. *Am. J. Physiol.* (2002) 282(4):G711- G719.

68. OPANASOPIT P, NISHIKAWA M, HASHIDA M: Factors affecting drug and gene delivery: effects of interaction with blood components. *Crit. Rev. Ther. Drug Carrier Syst.* (2002) 19(3):191-233.

•• Summarises several possibilities on the interaction of proteins and other macromolecules with blood components.

69. MA SF, NISHIKAWA M, KATSUMI H, YAMASHITA F, HASHIDA M: Cationic charge-dependent hepatic delivery of amidated serum albumin. *J. Control. Release* (2005) 102(3):583-594.

70. MIHARA K, OKA Y, SAWAI K, TAKAKURA Y, HASHIDA M: Improvement of therapeutic effect of human recombinant superoxide dismutase on ischemic acute renal failure in the rat via cationization and conjugation with polyethylene glycol. *J. Drug Target.* (1994) 2(4):317-321.

71. SCHALKWIJK J, VAN DEN BERG WB, VAN DE PUTTE LB, JOOSTEN LA, VAN DEN BERSSELAAR L: Cationization of catalase, peroxidase, and superoxide dismutase. Effect of improved intraarticular retention on experimental arthritis in mice. *J. Clin. Invest.* (1985) 76(1):198-205.

72. MORELL AG, GREGORIADIS G, SCHEINBERG IH, HICKMAN J, ASHWELL G: The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Biol. Chem.* (1971) 246(5):1461-1467.

73. ROGERS JC, KORNFELD S: Hepatic uptake of proteins coupled to fetuin glycopeptide. *Biochem. Biophys. Res. Commun.* (1971) 45(3):622-629.

74. CONNOLLY DT, TOWNSEND RR, KAWAGUCHI K, BELL WR, LEE YC: Binding and endocytosis of cluster glycosides by rabbit hepatocytes. Evidence for a short-circuit pathway that does not lead to degradation. *J. Biol. Chem.* (1982) 257(2):939-945.

75. NISHIKAWA M, OHTSUBO Y, OHNO J *et al.*: Pharmacokinetics of receptor-mediated hepatic uptake of glycosylated albumin in mice. *Int. J. Pharm.* (1992) 85(1):75-85.

76. NISHIKAWA M, MIYAZAKI C, YAMASHITA F, TAKAKURA Y, HASHIDA M: Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. *Am. J. Physiol.* (1995) 268(5 Pt 1):G849-G856.

77. OPANASOPIT P, SHIRAISHI K, NISHIKAWA M, YAMASHITA F, TAKAKURA Y, HASHIDA M: *In vivo* recognition of mannosylated proteins by hepatic mannose receptors and mannan-binding protein. *Am. J. Physiol.* (2001) 280(5):G879-G889.

78. SETT R, SARKAR HS, DAS PK: Pharmacokinetics and biodistribution of methotrexate conjugated to mannosyl human serum albumin. *J. Antimicrob. Chemother.* (1993) 31(1):151-159.

79. YABE Y, KOBAYASHI N, NISHIHASHI T *et al.*: Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. *J. Pharmacol. Exp. Ther.* (2001) 298(3):894-899.

80. JANSEN RW, MOLEMA G, CHING TL *et al.*: Hepatic endocytosis of various types of mannose-terminated albumins. What is important, sugar recognition, net charge, or the combination of these features. *J. Biol. Chem.* (1991) 266(5):3343-3348.

81. TERPSTRA V, VAN AMERSFOORT ES, VAN VELZEN AG, KUIPER J, VAN BERKEL TJ: Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler. Thromb. Vasc. Biol.* (2000) 20(8):1860-1872.

82. KOHLER G, MILSTEIN C: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* (1975) 256(5517):495-497.

83. KOZOWER BD, CHRISTOFIDOU-SOLOMIDOU M, SWEITZER TD *et al.*: Immunotargeting of catalase to the pulmonary endothelium alleviates oxidative stress and reduces acute lung transplantation injury. *Nat. Biotechnol.* (2003) 21(4):392-398.

84. NONAKA Y, IWAGAKI H, KIMURA T, FUCHIMOTO S, ORITA K: Effect of reactive oxygen intermediates on the *in vitro* invasive capacity of tumor cells and liver metastasis in mice. *Int. J. Cancer* (1993) 54(6):983-986.

• Demonstrates that subcutaneous catalase inhibits tumour metastasis to the liver, whereas subcutaneous SOD aggravates it.

85. SAFFORD SE, OBERLEY TD, URANO M, ST CLAIR DK: Suppression of fibrosarcoma metastasis by elevated expression of manganese superoxide dismutase. *Cancer Res.* (1994) 54(16):4261-4265.

86. KOGAWA K, MURAMATSU H, TANAKA M *et al.*: Enhanced inhibition of experimental metastasis by the combination chemotherapy of Cu-Zn SOD and adriamycin. *Clin. Exp. Metastasis* (1999) 17(3):239-244.

87. TAKENAGA M, IGARASHI R, OCHIAI A, MIZUSHIMA Y: Effect of lecithinized superoxide dismutase (PC-SOD) on experimental pulmonary metastasis in mice. *Free Radic. Biol. Med.* (1999) 26(9-10):1117-1125.

88. VAN ROSSEN ME, SLUITER W, BONTHUIS F, JEEKEL H, MARQUET RL, VAN EIJCK CH: Scavenging of reactive oxygen species leads to diminished peritoneal tumor recurrence. *Cancer Res.* (2000) 60(20):5625-5629.

• Shows that catalase significantly prevents peritoneal tumour recurrence in mice.

89. NISHIKAWA M, TAMADA A, HYOUDOU K *et al.*: Inhibition of experimental hepatic metastasis by targeted delivery of catalase in mice. *Clin. Exp. Metastasis* (2004) 21(3):213-221.

90. BAYON LG, IZQUIERDO MA, SIROVICH I, VAN ROOIJEN N, BEELEN RH, MEIJER S: Role of Kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver. *Hepatology* (1996) 23(5):1224-1231.

•• Clearly demonstrates that Kupffer cells are involved in the endogenous defence against hepatic metastasis of tumour cells.

91. RUSHFELDT C, SVEINBJORNSSON B, SELJELID R, SMEDSROD B: Early events of hepatic metastasis formation in mice: role of Kupffer and NK-cells in natural and interferon-γ-stimulated defense. *J. Surg. Res.* (1999) 82(2):209-215.

92. WENK J, BRENNEISEN P, WLASCHEK M *et al.*: Stable overexpression of manganese superoxide dismutase in mitochondria identifies hydrogen peroxide as a major oxidant in the AP-1-mediated induction of matrix-degrading metalloprotease-1. *J. Biol. Chem.* (1999) 274(36):25869-25876.

93. NISHIKAWA M, TAMADA A, KUMAI H, YAMASHITA F, HASHIDA M: Inhibition of experimental pulmonary metastasis by controlling biodistribution of catalase in mice. *Int. J. Cancer* (2002) 99(3):474-479.

94. ZHANG L, HELLSTROM KE, CHEN L: Luciferase activity as a marker of tumor burden and as an indicator of tumor response to antineoplastic therapy *in vivo*. *Clin. Exp. Metastasis* (1994) 12(2):87-92.

•• Shows the usefulness of reporter gene-expressing tumour cells in their detection *in vivo*.

95. VOOIJS M, JONKERS J, LYONS S, BERNS A: Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice. *Cancer Res.* (2002) 62(6):1862-1867.

96. RAY P, WU AM, GAMBHIR SS: Optical bioluminescence and positron emission tomography imaging of a novel fusion reporter gene in tumor xenografts of living mice. *Cancer Res.* (2003) 63(6):1160-1165.

97. DEMICHELI R, ABBATTISTA A, MICELI R, VALAGUSSA P, BONADONNA G: Time distribution of the recurrence risk for breast cancer patients undergoing mastectomy: further support about the concept of tumor dormancy. *Breast Cancer Res. Treat.* (1996) 41(2):177-185.

98. GOODISON S, KAWAI K, HIHARA J *et al.*: Prolonged dormancy and site-specific growth potential of cancer cells spontaneously disseminated from nonmetastatic breast tumors as revealed by labeling with green fluorescent protein. *Clin. Cancer Res.* (2003) 9(10 Pt 1):3808-3814.

99. MORRIS VL, KOOP S, MACDONALD IC *et al.*: Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. *Clin. Exp. Metastasis* (1994) 12(6):357-367.

100. CAMERON MD, SCHMIDT EE, KERKVLIET N *et al.*: Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. *Cancer Res.* (2000) 60(9):2541-2546.

101. BOUVY ND, MARQUET RL, JEEKEL J, BONJER HJ: Laparoscopic surgery is associated with less tumour growth stimulation than conventional surgery: an experimental study. *Br. J. Surg.* (1997) 84(3):358-361.

102. VAN DEN TOL PM, VAN ROSSEN EE, VAN EIJCK CH, BONTHUIS F, MARQUET RL, JEEKEL H: Reduction of peritoneal trauma by using nonsurgical gauze leads to less implantation metastasis of spilled tumor cells. *Ann. Surg.* (1998) 227(2):242-248.

103. MATOBA T, SHIMOKAWA H, NAKASHIMA M *et al.*: Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J. Clin. Invest.* (2000) 106(12):1521-1530.

• Provides the first evidence for the function of hydrogen peroxide as an endothelium-derived hyperpolarising factor.

Affiliation

Makiya Nishikawa[†1] PhD &
Mitsuru Hashida[2] PhD
[†]Author for correspondence
[1]Associate Professor, Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
Tel: +81 75 753 4580; Fax: +81 75 753 4614; E-mail: makiya@pharm.kyoto-u.ac.jp
[2]Professor, Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan