Table 2. Identified Proteins of HUVECs Markedly Changed in the Spot Signals by Treatment with VEGF

Definition	Official symbol[a]	Spot number[b]	GI number	Fold change	pI	Mw[c]	Coverage (%)	Classification
ACTB protein (actin, beta)	ACTB	15	15277503	1.59	5.55	40542	41	Cytoskeletal regulation
Aldehyde dehydrogenase 1	ALDH1A1	11	2183299	0.65	6.3	55438	19	Metabolism
Annexin 5	ANXA5	22	4502107	1.78	4.94	35972	23	Signal transduction
Annexin A2, isoform 2	ANXA2	24	16306978	1.51	7.57	38826	23	Signal transduction
Annexin I	ANXA1	19	4502101	1.53	6.57	38922	36	Signal transduction
Caldesmon 1 isoform 2	CALD1	4	4826657	1.56	6.18	62683	38	Cytoskeletal regulation
Catalase	CAT	10	4557014	0.61	6.9	59951	17	Antioxidant enzyme
Chain A, triosephosphate isomerase (Tim) (EC 5.3.1.1) complexed with 2-phosphoglycolic acid		27	999892	1.51	6.51	26812	45	Glycolytic enzyme
EH-domain containing protein 2	EHD2	8	57015322	1.67	5.95	61184	18	Unknown
Enolase 1	ENO1	13	4503571	2	7.01	47487	34	Glycolytic enzyme
Eukaryotic translation elongation factor 1 beta 2	EEF1B2	21	4503477	1.69	4.5	14922	27	Protein synthesis
Eukaryotic translation elongation factor 1 delta isoform 2 (guanine nucleotide exchange protein)	EEF1D	18	25453472	1.58	4.9	31219	26	Protein synthesis
Eukaryotic translation elongation factor 2	EEF2	1	4503483	1.53	6.41	96246	31	Protein synthesis
Gelsolin isoform b	GSN	3	38044288	1.86	5.58	80876	19	Cytoskeletal regulation
Heat shock 70 kDa protein 8 isoform 1	HSPA8	5	5729877	1.71	5.37	70186	42	Chaperone
Heat shock 90 kDa protein 1, beta	HSP90AB1	2	20149594	1.52	4.97	83554	44	Chaperone
Heterogeneous nuclear ribonucleoprotein A1 isoform a	HNRPA1	22	4504445	0.66	9.27	34291	35	RNA processing
Heterogeneous nuclear ribonucleoprotein C isoform b	HNRPC	16	4758544	0.63	5.1	32005	27	RNA processing
LIM and SH3 protein 1	LASP1	20	5453710	1.56	6.61	30104	28	Cytoskeletal reguration
MDS014 (RNA binding motif protein 8A)	RBM8A	34	10197630	0.57	6.46	18264	31	RNA processing
My032 protein (ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit d)	ATP5H	30	12002006	0.65	6.6	15821	70	Mitochondrial electron transport chain
Neighbor of COX4	COX4NB	29	5174615	0.52	5.92	24222	31	Mitochondrial electron transport chain
Peroxiredoxin 1	PRDX1	31	4505591	1.64	8.27	22328	42	Antioxidant enzyme
RAN protein (member RAS oncogene family)	RAN	28	32425497	0.61	7.16	25381	30	Signal transduction
Similar to Homo sapiens mRNA for KIAA0120 gene with GenBank accession number D21261.1		32	9956026	1.6	8.41	24438	84	Unknown
Splicing factor, arginine/serine-rich 3	SFRS3	35	4506901	0.64	11.64	19318	24	RNA processing
Succinate dehydrogenase complex, subunit A, flavoprotein precursor	SDHA	7	4759080	0.6	7.06	73687	30	Mitochondrial electron transport chain
TALDO1 protein (transaldolase 1)	TALDO1	17	17511894	1.57	6.36	37690	27	Pentose phosphate pathway
TKT protein (discoidin domain receptor family, member 2)	DDR2	6	31417921	1.61	8.02	50543	35	Pentose phosphate pathway
T-plastin polypeptide (plastin 3 [T form])	PSL3	9	190028	1.93	5.73	64289	46	Cytoskeletal regulation
TPM4-ALK fusion oncoprotein type 2		25	10441386	1.93	4.77	27571	23	Cytoskeletal regulation
TPMsk3 (tropomyosin 3)	TPM3	26	19072649	1.84	4.72	28908	23	Cytoskeletal regulation
Transgelin 2	TAGLN2	33	4507357	1.84	8.41	22377	84	Unknown
Translation elongation factor 1 alpha 1-like 14	EEF1A1L14	12	15277711	1.56	9.1	50457	23	Protein synthesis
Ubiquinol-cytochrome c reductase core protein I	UQCRC1	14	46593007	0.58	5.94	53281	8	Mitochondrial electron transport chain

a) From NCBI Entrez Gene. *b*) Numbers corresponding to those in Fig. 5. *c*) Molecular weight of protein.

VDAC1, an outer membrane protein of mitochondria, is a small and abundant pore-forming protein thought to form the major pathway for the movement of adenine nucleotides through the mitochondrial membrane. This protein is also known as a receptor for plasminogen kringle 5, which induces apoptosis of endothelial cells and regulates angiogenesis.[13] The direct relationship between VDAC1 and angiogenesis, however, is still unclear. Since HT1080-CM-stimulated endothelial cells highly express VDAC1 as well as malignant cells,[14] it might be a candidate as an effective target for cancer therapy.

ANXA2 is a member of the calcium-dependent phospholipid-binding protein family and known to play a role in the regulation of cellular growth and signal transduction. The expression of ANXA2 is known to be upregulated in various types of cancers such as pancreas, breast, and brain[15] and in tumor vasculature,[16] whereas its expression was downregulated in prostate cancer.[17] Since our results showed that ANXA2 expression was downregulated by HT1080-CM but upregulated by VEGF, HT1080-CM treatment may cause a response similar to prostate cancer. ANXA2 is also a cell surface receptor for angiostatin, which is a potent endoge-

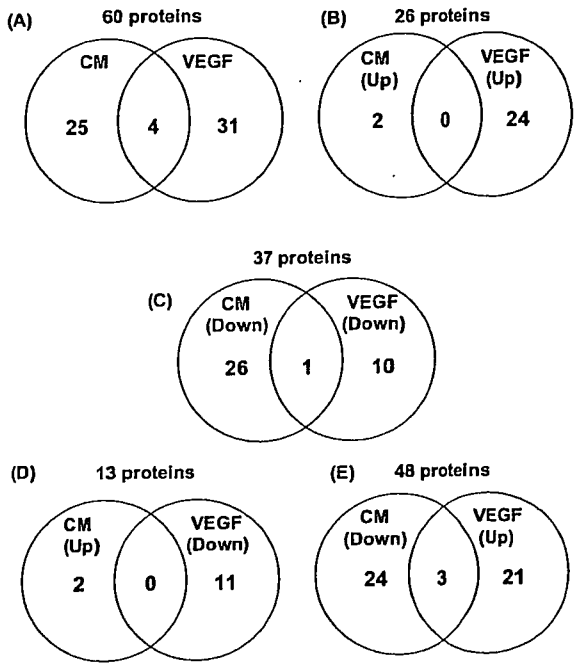

Fig. 6. Comparative Analysis of Protein Molecules in CM-Induced and VEGF-Induced Angiogenic Response

(A) Total 60 differentially expressed proteins; (B) proteins upregulated by both CM and VEGF stimulation; (C) those downregulated by both; (D) those upregulated by CM and downregulated by VEGF; (E) proteins downregulated by CM but upregulated by VEGF treatment. Numbers of the overlapping circles in the Venn diagrams shows the number of protein species differentially expressed in both types of stimulation.

nous angiogenesis inhibitor.[18] It was reported that angiostatin can suppress tumor growth and metastasis in various cancers.[19,20] It is possible that the downregulation of ANXA2 expression by HT1080-CM promotes CM-induced angiogenesis through the suppression of the effect of angiostatin.

ENO1 is a glycolytic enzyme and has been found to bind to an element in the c-myc promoter. Recently, it has been reported that ENO1 suppresses prostate cancer cell growth and induces neuroblastoma cell death.[21,22] Hence the downregulation of ENO1 induced by HT1080-CM might promote endothelial cell growth.

PLS3 is a member of an important actin-filament cross-linking protein family and controls cell motility by the maintenance of the actin cytoskeleton. This protein enhances actin base cell movement mediated by the Wiskott/Aldrich-syndrom protein (WASP).[23] Since WASP plays an important role in endothelial cell movement,[24] PLS3 might be associated with cellular motility during the HT1080-CM-regulated angiogenic response.

RAN is a small GTP-binding and Ras-related nuclear protein with an important role in the transport of RNA and proteins through the nuclear pore complex. This protein positively regulates the nuclear localization of PTEN, one of the important tumor-suppressor gene products, and nuclear PTEN is known to enhance apoptosis.[25] PTEN negatively controls the Akt signal-transduction pathway and is also closely associated with angiogenesis.[26] Downregulation of RAN by CM and VEGF might suppress the nuclear translocation of PTEN and promote angiogenesis.

In addition to these proteins, we identified some functional protein molecules that may be involved in CM-induced angiogenic response, such as glycolytic enzymes, protein degradation-related proteins, and antioxidant enzymes. Among them, UCHL1, which plays a role in proteasomal protein degradation, was reported to be reduced in metastatic melanoma and showed inhibitory effects on the metastasis of melanoma.[27]

As mentioned above, the differential expression of ALDH2, VDAC1, ANXA2, ENO1, PLS3, RAN, *etc.* in HT1080-CM-induced angiogenesis seems to be associated with cancer cell-mediated angiogenesis. Thus our data provide new information regarding the regulation of protein expression associated with tumor-induced angiogenesis and will be helpful in understanding the molecular mechanisms of tumor angiogenesis.

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Rapid communication

The contribution of phagocytic activity of liver macrophages to the accelerated blood clearance (ABC) phenomenon of PEGylated liposomes in rats

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Abstract

We earlier reported that PEGylated liposomes lose their long-circulating property when they are administered twice in the same animal within certain intervals. We recently proposed that anti-PEG IgM elicited by the first dose PEGylated liposomes selectively binds to the surface of a second dose, subsequently leading to substantial complement activation and complement-receptor mediated uptake of the second dose by hepatic Kupffer cells. In this study we found, by using a single-pass liver perfusion technique, that the first dose does not increase the intrinsic phagocytic activity of the Kupffer cells. It was also found that only serum obtained from rats that had received a first dose is able to enhance the hepatic uptake of test dose. The conditioned-serum-dependent hepatic uptake was completely abolished by pre-treatment of the serum at 56 °C for 30 min, which inhibits the complement activity. Conclusively, our results strongly support our earlier proposal that complement activation caused by anti-PEG IgM elicited by the first dose is a major cause of the initiation of the accelerated blood clearance of a subsequent dose PEGylated liposome in the ABC phenomenon.

Keywords: Accelerated blood clearance (ABC) phenomenon; PEGylated liposome, Polyethylene glycol (PEG); Liver perfusion; Complement system

1. Introduction

PEGylated liposomes, which have long-circulating properties, have been amply used as a drug carrier to improve the circulation lifetime of entrapped therapeutic agents [1]. It is believed that the PEG on the surface of liposomes, providing a steric barrier against the attachment of plasma proteins such as opsonins and recognition by the cells of the mononuclear phagocyte system, in turn, results in a decrease in the rate of clearance of the liposomes from blood circulation [2]. However, we and other researchers have observed that the first dose PEGylated liposomes cause a second dose, injected several days later, to lose its long-circulating properties and to accumulate extensively in the liver in mice, rats and rhesus monkeys (referred to as the "accelerated blood clearance (ABC) phenomenon") [3–6]. Based on our recent studies [7–9], we proposed the following tentative mechanism for the induction of the ABC phenomenon: anti-PEG IgM, produced by the spleen in response to the first dose, selectively binds to the PEG chains on a second dose administered several days later, and subsequently activates the complement system, one of the major opsonins [10–12], and enhances uptake of the second dose by the Kupffer cells [3,5,13].

Two phases can be distinguished in the ABC phenomenon [5,8]: the induction phase, following the first injection, during which the immune system is primed [reflected in the production of anti-PEG IgM], and the effectuation phase, following the second injection, during which the PEGylated liposomes are

Abbreviations: ABC, accelerated blood clearance; CHOL, cholesterol; HEPC, hydrogenated egg phosphatidylcholine; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; PEG, polyethylene glycol.

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rapidly cleared from the blood circulation [reflected in enhanced uptake by Kupffer cells]. Earlier studies indicated that Kupffer cells are at least partly responsible for the effectuation phase [3,5,9]. Thus, it is tempting to assume that liver macrophages acquire the ability to recognize and avidly take up PEGylated liposomes as the ABC phenomenon developed.

Therefore, in this study we investigated the issue of whether the injection of PEGylated liposomes increases the intrinsic, opsonin-independent, phagocytic activity of the Kupffer cells. Furthermore, we addressed the issue of whether the complement activation synergistically enhances the uptake of the second dose, resulting in a further increase in Kupffer cell-mediated liver uptake. To that end we employed a single-pass liver perfusion technique [14–16], because this method allows us to evaluate the issues under conditions at which the influence of serum is included or excluded.

2. Materials and methods

2.1. Materials and animal

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(poly-ethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by Nippon Oil and Fat (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Japan (Yokohama, Japan). All other reagents were of analytical grade.

Male Wistar rats (250–300g) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

2.2. Preparation of liposomes

PEGylated liposomes, composed of HEPC:CHOL:$mPEG_{2000}$-DSPE (1.85:1.0:0.15 molar ratio), were prepared as previously described [6]. The mean diameter of the prepared liposomes was determined by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The concentration of phospholipid was determined by colorimetric assay [17].

2.3. In situ a single-pass liver perfusion study

Rats had received an intravenous injection of PEGylated liposome (0.001 μmol/kg) or HEPES-buffered saline (pH 7.4) 5 days before were being subjected to the liver perfusion study described in the following. Perfused liver was prepared according to the method reported previously [14–16]. After a stabilization period of 10 min perfusion with Krebs–Ringer solution (pH 7.4) at a rate of 20 ml/min, radiolabeled test-dose PEGylated liposomes (4 mM in 7 ml of HEPES-buffered saline or serum) were infused via the portal vein at a constant rate (1 ml/min) for 7 min, following incubation with HEPES-buffered saline or serum at 37 °C for 15 min. After a 3-min wash with liposome-free perfusate, the liver was removed and weighed, and the radioactivity in whole liver was measured according to the method reported previously [18]. The uptake of liposomes was expressed as extraction (the percentage uptake of liposomes by the liver). Satisfactory liver viability was assessed on the basis of a bile flow rate >1 μl/min/g liver. Four to five rats were used in each group.

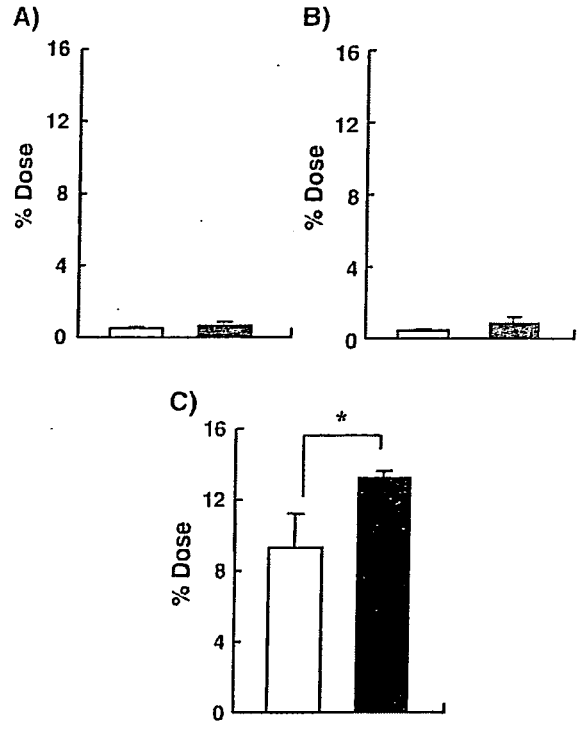

Fig. 1. Hepatic uptake of radiolabeled test-dose PEGylated liposomes in a single-pass liver perfusion. PEGylated liposomes (0.001 μmol phospholipids/kg) or HEPES-buffered saline was intravenously injected into rats. Five days later, a single-pass liver perfusion was carried out with pre-treated rats, using differently prepared radiolabeled test dose PEGylated liposomes. Open columns represent livers of rats (n=4–5) that had received HEPES-buffered saline. Filled columns represent livers of rats (n=4–5) that were pre-dosed with PEGylated liposomes. (A) test-dose liposomes were incubated with HEPES-buffered saline. (B) test-dose liposomes were incubated with serum from rats that had only received HEPES-buffered saline. (C) test-dose liposomes were incubated with serum from rats pre-dosed with PEGylated liposome. Each value represents the mean±S.D. of 4 or 5 separate experiments. * $p<0.05$.

2.4. Statistics

All values are expressed as the mean±S.D. Statistical analysis was performed with a two-tailed unpaired t test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $p<0.05$.

3. Results and discussion

3.1. Effect of pre-dose of PEGylated liposomes on the intrinsic phagocytic activity of liver macrophages towards test-dose PEGylated liposomes

To study the effect of pre-dose PEGylated liposomes on the intrinsic phagocytic activity of liver macrophages, we perfused rat livers with a pre-dose PEGylated liposomes (0.001μmol

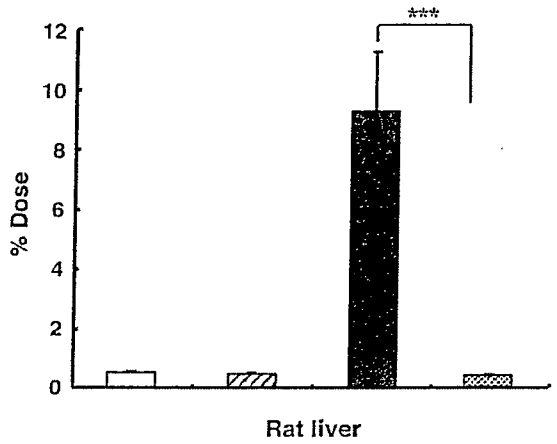

Fig. 2. Effect of heat-inactivation of serum on the serum-dependent hepatic uptake of test-dose PEGylated liposomes. Radiolabeled test-dose PEGylated liposomes were infused for single-pass perfusion into the livers of rats that had received HEPES-buffered saline 5 days before. Open column; test-dose liposomes pre-incubated with HEPES-buffered saline. Hatched column; test-dose liposomes incubated with serum from rats that were pre-dosed with HEPES-buffered saline. Filled column; test-dose liposomes incubated with serum from rats pre-dosed with PEGylated liposome. Dotted column; test-dose liposomes incubated with heat-treated serum (30 min 56 °C) from rats pre-dosed with PEGylated liposomes. Each value represents the mean±S.D. of 4 or 5 separate experiments. *** $p<0.005$.

phospholipid/kg), that is capable of causing the ABC phenomenon at a maximal level [9,13]. In the absence of serum in the perfusate, no enhancement in hepatic uptake of radiolabeled test-dose PEGylated liposomes was observed (Fig. 1A). This result obviously shows that the intravenous injection of PEGylated liposomes 5 days before did not increase the intrinsic phagocytic activity of the liver macrophages.

3.2. Effect of opsonization with rat serum on the uptake of test-dose PEGylated liposomes by liver macrophages

The test-dose liposomes were infused in the liver following incubation at 37 °C for 15 min with two types of sera. The serum obtained from rats that had received HEPES-buffered saline did not enhance the uptake of test-dose liposomes by the livers of rats that had received either PEGylated liposomes or HEPES-buffered saline (Fig. 1B). By contrast, serum obtained from rats that were pre-dosed with PEGylated liposomes significantly enhanced the hepatic uptake of test-dose liposomes in the livers of rats that were pre-dosed with either PEGylated liposomes or HEPES-buffered saline (Fig. 1C). These indicate that the first-dose PEGylated liposome induces production of serum factor(s), which is/are responsible for enhanced hepatic uptake of test-dose PEGylated liposomes.

It should be noted that the level of hepatic uptake of test-dose liposomes was significantly higher in the liver of pre-dosed rats than in the liver of rats that had only received HEPES-buffered saline (Fig. 1C). This suggests that the pre-dose PEGylated liposome augments the macrophages' response to properly opsonized liposomes. It is likely that the ABC phenomenon occurs as a result of an immune response induced by the first dose [7–9,19]. Such immunological responses may result in the production of a variety of cytokines and/or chemokines [20,21]. The cytokines/chemokines thus produced might stimulate the liver macrophages directly and thereby induce the expression of receptors such as complement receptors and Fc receptors, related to phagocytosis and/or endocytosis of opsonized materials.

3.3. Heat-sensitivity of the serum factor(s) causing the serum-dependent hepatic uptake of test-dose PEGylated liposomes

To identify serum factors that are involved in the serum-dependent hepatic uptake (Fig. 1), the serum obtained from rats pre-dosed with PEGylated liposome was pre-treated by heating at 56 °C for 30 min, which is known to inhibit all complement activity. Following incubation for 30 min at 37 °C with this heat-treated serum, test-dose liposomes were infused in the livers of rats that had received HEPES-buffered saline. The heated serum (56 °C, 30 min) completely abolished the serum-dependent hepatic uptake of test-dose liposomes (Fig. 2).

We recently proposed the tentative mechanism for the induction of the ABC phenomenon [7–9]. Anti-PEG IgM, induced by the first injection of PEGylated liposomes, plays a key role in the mechanism. IgM has a strong potential to activate the complement system and consequently enhances the uptake of foreign materials via complement receptor-mediated endocytosis or phagocytosis, but by itself it has no ability to promote endocytosis or phagocytosis directly. It is well known that the complement system loses activity when treated at 56 °C for 30 min [12]. The serum-dependent uptake of test dose by liver was virtually abolished by treatment of the serum at 56 °C for 30 min (Fig. 2). This finding proves that complement activation, probably triggered by selective binding of anti-PEG IgM to test-dose PEGylated liposome [7–9], is responsible for the ABC phenomenon. This is supported by previous findings of Dams et al. [3] showing that the accelerated blood clearance of PEGylated liposomes is mediated by a soluble heat-labile (56 °C, 30 min) serum factor(s). Therefore, we could confirm that the anti-PEG IgM-mediated complement activation induced by the second dose PEGylated liposome is the major cause of the induction of the accelerated blood clearance of PEGylated liposome. Nevertheless, we can not exclude an alternative explanation that the additive or synergistic effect of activated liver macrophages (Fig. 1C) and complement activation enhanced uptake of the test dose PEGylated liposome in the ABC phenomenon.

4. Conclusion

This paper highlights that first-dose PEGylated liposomes do not increase the intrinsic phagocytic activity of Kupffer cells and supports the notion that complement activation is essential for induction of the accelerated blood clearance of second-dose PEGylated liposomes.

Acknowledgments

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