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Potassium bromate treatment predominantly causes large deletions, but not GC > TA transversion in human cells

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Received 21 October 2006; received in revised form 24 February 2007; accepted 28 February 2007

Available online 4 March 2007

Abstract

Potassium bromate (KBrO₃) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells *in vitro*. The proposed genotoxic mechanism for KBrO₃ is oxidative DNA damage. KBrO₃ can generate high yields of 8-hydroxydeoxyguanosine (8OHdG) DNA adducts, which cause GC > TA transversions in cell-free systems. In this study, we investigated the *in vitro* genotoxicity of KBrO₃ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (TK) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that KBrO₃ directly yielded DNA damages including DNA double strand breaks (DSBs). KBrO₃ also induced MN and TK mutations concentration-dependently. At the highest concentration (5 mM), KBrO₃ induced MN and TK mutation frequencies that were over 30 times the background level. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the TK locus. Ionizing-irradiation exhibited similar mutational spectrum in our system. These results indicate that the major genotoxicity of KBrO₃ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed. To better understand the genotoxic mechanism of KBrO₃, we analyzed gene expression profiles of TK6 cells using Affymetrix Genechip. Some genes involved in stress, apoptosis, and DNA repair were up-regulated by the treatment of KBrO₃. However, we could not observe the similarity of gene expression profile in the treatment of KBrO₃ to ionizing-irradiation as well as oxidative damage inducers.

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Keywords: Potassium bromate (KBrO₃); TK-mutation; Loss of heterozygosity (LOH); 8-Hydroxydeoxyguanosine (8OHdG); Gene expression profile

1. Introduction

Potassium bromate (KBrO_3) is used as in bread making a flour improver and in the production of fish-pastes. The EU countries now prohibit its use as a food additive because of its carcinogenicity. Japan and the USA, however, permit its use in bread making on the condition that it never remains in the final product. KBrO_3 causes tumors, especially in kidney, in rats, and mice after long-term oral administration in drinking water [1–3]. KBrO_3 is also genotoxic. It is positive in *in vitro* genotoxicity tests – including the bacterial reverse mutation assay [1], the chromosomal aberration test conducted in Chinese hamster cells [4], and the mouse lymphoma assay [5] – and *in vivo* in the micronucleus test (MN) [6,7].

It has been proposed that KBrO_3 induces tumors through the production of oxidative damage to DNA. Oxidative DNA damage can cause mutations that contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, thereby leading to tumorigenesis [8,9]. 8-Hydroxydeoxyguanosine (8OHdG) is the main form of oxidative DNA damage induced by KBrO_3 [10]. It primarily causes GC>TA transversions (as a result of the pairing of 8OHdG with A) and is believed to be responsible for mutagenesis, carcinogenesis, and aging [11,12]. KBrO_3 increases 8OHdG DNA adducts *in vivo* and *in vitro* [13–15]. However, KBrO_3 induces mutations weakly in microbial mutation assays and the *Hprt* mutation assay in mammalian cells, while it induces chromosome aberrations strongly both *in vivo* and *in vitro* [1,16,17]. These findings raise the question of whether 8OHdG is required for the mutagenic process involved in KBrO_3 -induced carcinogenesis.

In the present study, we examined the genotoxic properties of KBrO_3 using the comet assay (COM), the MN test, and thymidine kinase (*TK*) gene mutation assays in human lymphoblastoid TK6 cells [18]. Unlike the X-linked hemizygous *HPRT* gene mutation assay, the *TK* mutation assay can detect not only point mutations, but also large scale chromosomal deletions, recombinations, and aneuploidy [19–21]. Most of the genetic changes observed in *TK* mutants occur in human tumors and are presumed relevant to carcinogenesis. We analyzed the *TK* mutants induced by KBrO_3 at the molecular level and investigated what kind of mutation predominated. We also profiled global gene expression in TK6 cell exposed to KBrO_3 using Affymetrix GeneChip[®] Expression analysis to understand the genotoxic mechanism of KBrO_3 .

2. Materials and methods

2.1. Cell culture, chemicals, and treatment

The TK6 human lymphoblastoid cell line has been described previously [22]. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JR Biosciences, Lenexa, KS), 200 $\mu\text{g}/\text{ml}$ sodium pyruvate, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere with 100% humidity. KBrO_3 (CAS No.7758-01-2) was purchased from Wako Pure Chemical Co. (Tokyo) and dissolved in RPMI medium just before use.

We prepared 20 ml aliquots of cell suspension at a concentration of 5.0×10^5 cells/ml in 50 ml polystyrene tubes. Different concentrations of KBrO_3 were added to the tubes, which were then placed on a platform shaker and incubated at 37 °C for 4 h with gentle shaking. At the end of the treatment period, the cell cultures were centrifuged, washed once, and re-suspended in fresh medium. We cultured them in new flasks for the MN assay and *TK* gene mutation assay, or diluted them for plating for survival estimates.

2.2. Genotoxicity assays

After treating cells with KBrO_3 , we prepared slides for conducting the alkaline and neutral COM assay. The alkaline COM assay was performed as previously reported [23]. For the neutral COM assay, the slide was electrophoresed with chilled neutral solution (pH 8) containing of 90 mM Tris, 2 mM Na_2EDTA , and 90 mM boric acid according to the method by Wada et al. [24]. The COM slides were stained with SYBER green (Molecular Probes, Eugene, OR) and observed by an Olympus model BX50 fluorescence microscope. At least 50 cells were captured by CCD camera, and tail length of the comet was measured. The relationship between KBrO_3 treatment and migration was statistically analyzed by the Dunnett test [25].

We prepared the MN test samples 48 h after treatment, as previously reported [23]. Briefly, approximately 10^6 cells suspended in hypotonic KCl solution were incubated for 10 min at room temperature, fixed twice with ice-cold methanol containing 25% acetic acid, then re-suspended in methanol containing 1% acetic acid. A drop of the suspension was placed on a clean glass slide and air-dried. The cells were stained with 40 $\mu\text{g}/\text{ml}$ acridine orange solution and immediately observed with the aid of an Olympus model BX50 fluorescence microscope equipped with a U-MWBV band pass filter. At least 1000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between non-treated and treated cells were statistically analyzed by Fisher's exact test [26].

We prepared the *TK* gene mutation assay samples 3 days after treatment. We seeded cells from each culture into 96-well plates at 40,000 cells/well in the presence of 3.0 $\mu\text{g}/\text{ml}$ trifluo-

rothymidine (TFT). We also plated 1.6 cells/well without TFT to determine plating efficiency. All plates were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. After 14 days, we scored colonies on the PE plates and the normal-growing (NG) *TK* mutants on the TFT plates, then re-fed the plates containing TFT with fresh TFT, incubated them for an additional 14 days, and scored them for slow-growing (SG) *TK* mutants. Mutation frequencies, relative survival (RS), and relative suspension growth (RSG) were calculated as previously described [23]. The data of mutant frequencies were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson–Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [27].

2.3. LOH analysis of *TK* mutations by polymerase chain reaction (PCR)

To avoid analyzing identical mutants, we performed an additional *TK* mutation assay and isolated *TK* mutants from independent culture after a 4 h treatment with 2.5 mM KBrO₃. We confirmed the phenotype of the *TK* mutant clones by re-challenging them with TFT medium. We also determined the growth rate of the clones and confirmed whether they were NG or SG mutants.

Genomic DNA was extracted from the *TK* mutant cells and used as a template for PCR. We conducted the PCR-based LOH analysis of the human *TK* gene as described previously [28]. A set of primers was used to each amplify the parts of exons 4 and 7 of the *TK* gene that is heterozygous for frame shift mutations. A third primer set for amplifying parts of the β -globin was also used as the internal control. We applied quantitative-multiple PCR for co-amplification of the three regions. The PCR products were analyzed with an ABI310 genetic analyzer (PE Biosystems, Chiba, Japan), and were classified into "no LOH", "hemizygous (hemi-) LOH", or "homozygous (homo-) LOH". To determine the extent of the LOH, we analyzed 10 microsatellite loci on chromosome 17q by PCR-based LOH analysis [28]. The results were processed by GenoTyper™ software (PE Biosystems, Chiba, Japan) according to the manufacturer's guidelines.

2.4. Gene expression analysis

Total RNA was isolated from the TK6 cells after 4 h treatment with 2.5 mM KBrO₃ and was purified by RNeasy columns (Qiagen, Valencia, CA). We conducted a single cDNA synthesis, cRNA labeling, and cRNA fragmentation according to the manufacturer's recommendations (Affymetrix Inc., Santa Clara, CA) and employed Affymetrix GeneChip Expression analysis. The hybridization mixture for each sample was hybridized to an Affymetrix U133A human genome array. We processed the scanned data using Microarray Suite Software Version 5.0 (Affymetrix Inc., Santa Clara, CA) and imported the data into GeneSpring software (Silicon Genetics, Redwood City, CA). Signal intensity was normalized by per-gene and

per-chip, and the ratios were calculated by normalizing KBrO₃ sample to the corresponding control sample. We used intensity-dependent (step-wise) selection of significant changes with higher cut-off value for lower signal intensity (1.75-, 2.0-, 2.25-, 2.5-, and 3.5-fold for genes intensity range of >1000, 500–1000, 100–500, 50–100, and 10–50, respectively), and up-regulated genes with a presence call in KBrO₃ sample, whereas down-regulated genes with a presence call in the control sample.

3. Results

3.1. Cytotoxicity and genotoxicity of KBrO₃

KBrO₃ exerted strong and concentration-dependent cytotoxicity in TK6 cells (Fig. 1). It induced approximately 50% cytotoxicity (51% RSG and 44% RS) at 2.5 mM. To investigate whether KBrO₃ directly causes DNA damage, we conducted the COM assay. Induction of COM tail after the treatment of in alkaline version was statistically significant 2.5 and 5 mM. In the neutral COM assay, the induction was observed from the lower concentration (Fig. 1). Because the neutral COM is thought to be associated with DNA double strand breaks (DSBs) [29], this result indicates that KBrO₃ directly causes DNA damage including DSBs. KBrO₃ also induced MN and *TK* mutation in a concentration-dependent manner and their inductions were statistically significant (Fig. 1). At the maximum concentration, it induced both MN and *TK* mutation frequencies about 30 times the control values. Two distinct phenotypic classes of *TK* mutants were generated: NG mutants grew at the same rate as the wild type (doubling time 13–17 h), and SG mutants grew at a slower rate (doubling time > 21 h). NG mutants result from intragenic mutations, while SG mutants result from gross changes (extending beyond the *TK* gene) [20]. KBrO₃ predominantly induced SG mutants (Fig. 1), implying that KBrO₃ treatment predominantly causes gross structural changes, but not small genetic alterations such as point mutations.

3.2. Molecular analysis of *TK* mutants

The *TK* mutants were randomly isolated from independent cultures treated with 2.5 mM KBrO₃ for 4 h. Table 1 shows the cytotoxicity (RSG), mutation frequency, and proportion of SG mutants induced by KBrO₃. We subjected 40 induced mutants to LOH analysis. Of those, 32 (80%) were SG mutants, which corresponded closely to the percentage of SG mutants induced in the assay (74.1%), indicating that the result of LOH analysis reflected the character of the induced

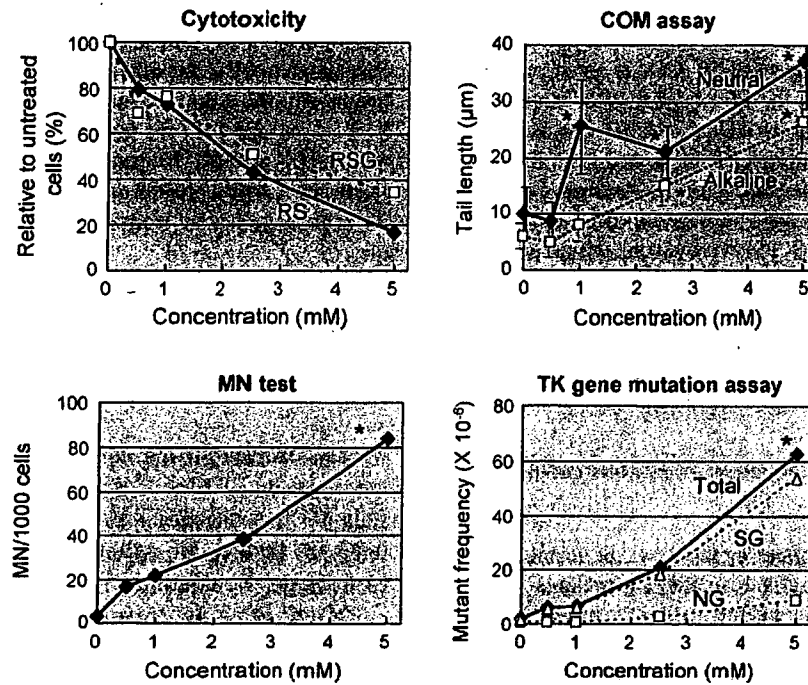


Fig. 1. Cytotoxic (relative survival, RS; relative suspension growth, RSG) and genotoxic responses (COM assay, MN test, and TK gene mutation assay) of TK6 cells treated with KBrO₃ for 4 h. Asterisk (*) statistically significant in Dunnett's test ($P < 0.05$) in COM assay, and in both pair-wise comparison and trend test ($P < 0.05$) in MN test and TK gene mutation assay.

mutations. Table 1 also shows the results of LOH analysis of the induced and spontaneously occurring mutants. The result of molecular analysis of spontaneous TK mutants was reported previously [21]. We classified the mutants into three types: non-LOH, hemizygous LOH (hemi-LOH), and homozygous LOH (homo-LOH). In general, hemi-LOH is resulted by deletion and homo-LOH is by inter-allelic homologous recombination [20]. Among the KBrO₃-induced mutants, 63% of NG mutants and 84% of SG mutants were hemi-LOH. In spontaneous mutants, on the other hand, majority of NG and SG mutants were non-LOH and homo-LOH, respectively. These results indicated that KBrO₃ predominantly induced large dele-

tions. We previously reported the mutational spectra of TK mutants in TK6 cells that treated with the alkylating agent ethylmethane sulfonate (EMS), or X-irradiated [20,21]. Fig. 2 shows the comparison of the mutational spectra of spontaneous and induced TK mutants by EMS, X-irradiation, and KBrO₃. The mutation spectrum induced by KBrO₃ was similar to that induced by X-radiation (which also induces LOH, predominantly via deletion [21]) but not by EMS. The majority of the mutations induced by KBrO₃ were large deletions, but not point mutations.

Fig. 3 shows the regions of LOH and the distribution of spontaneous, X-ray-induced, and KBrO₃-induced

Table 1
Cytotoxic and mutational responses to KBrO₃, and the results of LOH analysis of normally growing (NG) and slowly growing (SG) TK mutants

Treatment	Cytotoxic and mutational response			LOH analysis at TK gene (%)			
	RSG (%)	MF ($\times 10^{-6}$)	% SG	Number	Non-LOH	Hemi-LOH	Homo-LOH
Spontaneous ^a	100	2.19	56	56			
NG mutants				19	14 (74)	3 (16)	2 (11)
SG mutants				37	0 (0)	9 (24)	28 (76)
KBrO ₃ (2.5 mM)	51	29.4	74	39			
NG mutants				8	3 (37)	5 (63)	0 (0)
SG mutants				31	1 (3)	27 (84)	4 (13)

^a Data from Zhan et al. [22].

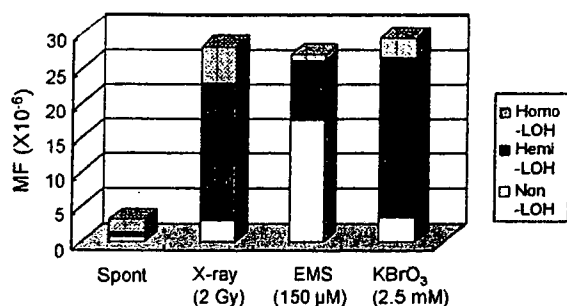


Fig. 2. *TK* mutation spectra in untreated, X-ray-treated (2 Gy), EMS-treated (150 μ M, 4 h), and KBrO₃-treated (2.5 mM, 4 h) TK6 cells. The fraction of each mutational event was calculated by considering the ratio of NG to SG mutants and the results of molecular analysis (Table 1). The data for all but the KBrO₃ treatments were taken from our previous paper [20].

LOH mutants. KBrO₃ predominantly induced hemi-LOH, the result of large interstitial and terminal deletions, which we also frequently observed in the X-ray-induced LOH mutants. These results indicate that the genetic changes induced by KBrO₃ were similar to those induced by X-rays.

3.3. Gene expression analysis

Table 2 lists the genes that significantly increased expression following exposure to 2.5 mM KBrO₃. These genes are involved in stress response (6 genes), cell growth and DNA repair (19 genes), immune response (3 genes), apoptosis (3 genes), signal transduction (10 genes), transcription regulation (10 genes), chromo-

some organization (2 genes), protein modification (7 genes), energy metabolism (6 genes), lipid metabolism (2 genes), purine biosynthesis (3 genes), and unclassified functions (42 genes). Table 3 shows the genes whose expression was suppressed by the treatment. The number of up-regulated genes was greater than the number of down-regulated genes.

4. Discussion

KBrO₃ is a complete carcinogen, possessing both initiating and promoting activities in rodents [1]. While it shows clear positive responses in the COM assay, MN test, and chromosome aberration test using mammalian cells [4,14,17], the mutagenic potential of KBrO₃ in bacteria and the *Hprt* assay in Chinese hamster cells is weak or negative [1,14,17,30]. In our present study, KBrO₃ treatment strongly induced *TK* gene mutations. The reason we observed the induction of gene mutations and others did not is that KBrO₃ induces detectable mutagenicity in the *TK* gene but are only weakly mutagenic or non-mutagenic in the *Hprt* gene and in microbial assays [20]. The lower mutation frequency in the *Hprt* gene is due to the low recovery of large deletions, which are not detected because they are lethal. KBrO₃ is positive in mouse lymphoma cell assays that target the *Tk* gene [5]. In in vivo genotoxicity tests, KBrO₃ strongly induces MN in male ddY mice but is only weakly mutagenic in the *gpt* mutation assay in transgenic mice, which mainly detects point mutations and small deletions [31]. These results indicate that the property of genotoxicity

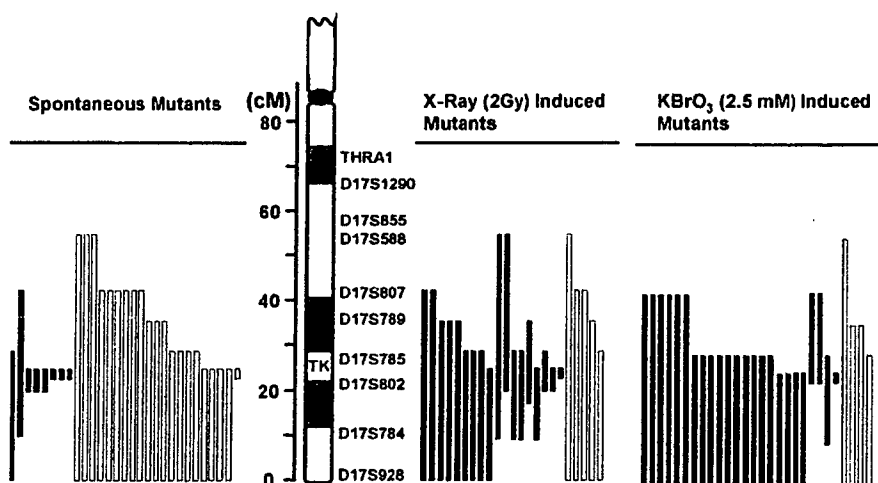


Fig. 3. The extent of LOH at the *TK* locus of TK6 cells that were untreated, X-ray-irradiated (2 Gy), or exposed to KBrO₃ (2.5 mM, 4 h). We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human *TK* locus maps to 17q23.2. Open and closed bars represent homozygous LOH and hemizygous LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 28 LOH mutants (4 NG and 24 SG). The data on spontaneous and X-ray-induced mutants were taken from our previous paper [20].

Table 2

Genes whose expression was up-regulated by KBrO₃ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Stress response	CAT	2.77	Catalase
	DNAJC7	2.33	DnaJ (Hsp40) homolog, subfamily C, member 7
	FKBP5	2.87	FK506 binding protein 5
	HSPA8	3.02	Heat shock 70 kDa protein 8
	HSPCB	3.21	Heat shock 90 kDa protein 1, beta
	HSPD1	1.83	Heat shock 60 kDa protein 1
DNA repair, cell cycle, cell growth	BUB1	4.51	BUB1 budding uninhibited by benzimidazoles 1 homolog
	CCND2	5.08	Cyclin d2
	CCT2	3.33	Chaperonin containing TCP1, subunit 2 (beta)
	DKC1	2.37	Dyskeratosis congenita 1, dyskerin
	ENO1	2.10	Enolase 1 (alpha)
	HMGB1	2.16	High-mobility group box 1
	MAPRE1	2.32	Microtubule-associated protein, RP/EB family, member 1
	NME1	2.00	Non-metastatic cells 1, protein (NM23A) expressed in
	NOLC1	2.99	Nucleolar and coiled-body phosphoprotein 1
	NRAS	2.54	Neuroblastoma RAS viral (v-ras) oncogene homolog
	p21	3.22	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	PPP2R1B	2.45	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform
	RAD21	2.34	RAD21 homolog
	RBBP4	2.00	Retinoblastoma binding protein 4
RHOA	1.77	ras homolog gene family, member A	
SRPK1	2.75	SFRS protein kinase 1	
SSR1	2.66	Signal sequence receptor, alpha	
Immune response	ARHGDI3	1.78	Rho GDP dissociation inhibitor (GDI) beta
	HLA-DRA	2.16	Major histocompatibility complex, class II, DR alpha
	IL2RG	2.43	Interleukin 2 receptor, gamma
Apoptosis	BCLAF1	6.42	BCL2-associated transcription factor 1
	FXR1	3.32	Fragile X mental retardation, autosomal homolog 1
	VDAC1	1.94	Voltage-dependent anion channel 1
Signal transduction	ANP32A	3.20	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
	OGT	2.74	O-linked <i>N</i> -acetylglucosamine (GlcNAc) transferase
	PIP5K1A	4.25	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
	PLEK	2.95	Pleckstrin
	PTPN11	2.61	Protein tyrosine phosphatase, non-receptor type 11
	SPTLC1	2.62	Serine palmitoyltransferase, long chain base subunit 1
	SRPR	2.52	Signal recognition particle receptor
Transcription regulation	CDC5L	4.37	CDC5 cell division cycle 5-like
	HNRPC	4.40	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	NO NO	2.68	Non-POU domain containing, octamer-binding
	POLR1C	2.67	Polymerase (RNA) I polypeptide C, 30 kDa
	PRPF4	2.51	PRP4 pre-mRNA processing factor 4 homolog
Chromosome organization	CBX5	2.68	Chromobox homolog 5 (HP1 alpha homolog, Drosophila)
Protein modification	CANX	2.56	Calnexin
	COPA	6.55	Coatomer protein complex, subunit alpha
	EIF2S3	2.40	Eukaryotic translation initiation factor 2, subunit 3 gamma
	EIF4B	2.86	Eukaryotic translation initiation factor 4B
	RANBP2	3.96	RAN binding protein 2
	SEC23IP	2.67	SEC23 interacting protein

Table 2 (Continued)

	Gene symbol	Ratio	Gene title
Energy pathway	AFURS1	2.83	ATPase family homolog up-regulated in senescence cells
	CYB5-M	2.54	Cytochrome <i>b5</i> outer mitochondrial membrane precursor
	TOMM22	3.07	Translocase of outer mitochondrial membrane 22 homolog
Lipid metabolism	HMGCS1	2.58	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
	SCD	2.56	Stearoyl-CoA desaturase
Purine biosynthesis	ENTPD1	2.36	Ectonucleoside triphosphate diphosphohydrolase 1
	GART	2.64	Phosphoribosylglycinamide formyltransferase
	PAICS	1.79	Phosphoribosylaminoimidazole carboxylase
Unclassified	BANF1	2.77	Barrier to autointegration factor 1
	BAT1	1.95	HLA-B associated transcript 1//HLA-B associated transcript 1
	C1orf16	2.37	Chromosome 1 open reading frame 16
	CALU	2.40	Calumenin
	DAZAP2	2.57	DAZ associated protein 2
	DDX18	2.34	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
	DHX9	9.37	DEAH (Asp-Glu-Ala-His) box polypeptide 9
	EXOSC2	3.03	Exosome component 2
	FLJ10534	2.07	Hypothetical protein FLJ10534
	FLJ10719	2.42	Hypothetical protein FLJ10719
	FLJ12973	2.76	Hypothetical protein FLJ12973
	GANAB	2.07	Glucosidase, alpha; neutral AB
	HEM1	2.37	Hematopoietic protein 1
	IGHM	2.76	Anti-HIV-1 gp120 V3 loop antibody DO142-10 light chain variable region
	IGKC	3.15	Anti-rabies virus immunoglobulin rearranged kappa chain V-region
	LN7C	3.51	lin-7 homolog C (<i>C. elegans</i>)
	LOC54499	2.31	Putative membrane protein
	M6PR	3.59	Mannose-6-phosphate receptor
	MGC8902	2.27	Hypothetical protein MGC8902/
	MOBK1B	2.67	MOB1, Mps one binder kinase activator-like 1B (yeast)
	NS	2.15	Nucleostemin
	NUSAP1	3.25	Nucleolar and spindle associated protein 1
	OK/SW-cl.56	1.85	Beta 5-tubulin
	OPRS1	2.76	Opioid receptor, sigma 1
	PEG 10	2.50	Paternally expressed 10
	PEX19	2.34	Peroxisomal biogenesis factor 19
	PGK1	2.11	Phosphoglycerate kinase 1
	RPE	2.35	Ribulose-5-phosphate-3-epimerase
	SDBCAG84	3.16	Serologically defined breast cancer antigen 84
	SMU1	2.70	smu-1 suppressor of mec-8 and unc-52 homolog (<i>C. elegans</i>)
	TAGLN2	2.03	Transgelin 2
	UBC	2.65	Ubiquitin C
	XPNPEP1	2.84	X-prolyl aminopeptidase
	YWHAE	6.39	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
	YWHAZ	2.50	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

of KBrO_3 predominantly causes gross structural changes rather than small genetic changes such as point mutations.

KBrO_3 generates high yields of 8OHdG DNA adducts, which is a marker of oxidative DNA damage widely used as a predictor of carcinogenesis [10]. 8OHdG has been reported to be highly mutagenic in some experiments. In cell-free system, 8OHdG induced

mutation by misincorporating adenine instead of cytosine [12]. Artificially incorporated 8OHdG at specific codons in a shuttle vector system efficiently induced GC>TA transversions in mammalian cells and *E. coli* [8,32,33]. In mammalian gene mutation assays in vitro and in vivo, however, the relationship between the accumulation of 8OHdG and the induction of GC>TA transversion has not been clear. Takeuchi et al.

Table 3
Genes whose expression was down-regulated by KBrO₃ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Cell cycle, cell growth	FH	0.51	Fumarate hydratase
	MYC	0.55	v-myc myelocytomatosis viral oncogene homolog
Signal transduction	DUSP2	0.37	Dual specificity phosphatase 2
	RRBP1	0.39	Ribosome binding protein 1 homolog 180 kDa
	TBL3	0.43	Transducin (beta)-like3
Transcription regulation	CITED2	0.45	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
	KIAA1196	0.43	KIAA1196 protein
	TZFP	0.39	Testis zinc finger protein
Chromosome organization	H1FX	0.14	H1 histone family member X
Protein modification	CLTB	0.43	Clathrin, light polypeptide (Lcb)
Energy pathway	FDX1	0.45	Ferredoxin 1
	QPRT	0.41	Quinolate phosphoribosyltransferase
	SLC39A4	0.43	Solute carrier family 39 (zinc transporter), member 4
Unclassified	BTBD2	0.35	BTB (POZ) domain containing 2
	LOC339229	0.44	Hypothetical protein LOC339229
	MGRN1	0.44	Mahogunin, ring finger 1
	MRP63	0.41	Mitochondrial ribosomal protein 63
	PHLDA1	0.43	Pleckstrin homology-like domain, family A, member 1
	PTPLA	0.37	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a
	SPATA2	0.45	Spermatogenesis associated 2

examined the mutagenicity of a hydroxyl radical generator, *N,N'*-bis (2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic diimide (NP-III). Although NP-III highly produced 8OHdG upon irradiation with UV in V79 cells, the frequency of *Hprt* gene mutation was not significantly induced [34]. Molecular analysis demonstrated the no association of induction of 8OHdG with GC>TA transversion in the *Hprt* mutants [35]. 8OHdG is mainly removed by Ogg1 protein in a manner of the base excision repair (BER) pathway. Arai et al. investigated the relationship between the accumulation of oxidative DNA damage and the induction of gene mutation using *Ogg1* deficient transgenic mice [36]. Although the 8OHdG level in kidneys of the *Ogg1* deficient mice increase 200 times of the control level after 4 weeks' KBrO₃ treatment, the mutation frequency in the transgenic *gpt* gene was induced by less than 10 times of the control level. The molecular analysis revealed that the fraction of GC>TA transversions did not specifically increase. These results suggest that 8OHdG-mediated base substitutions do not mainly contribute to the mutagenic process involved in KBrO₃-induced carcinogenesis. Other genotoxic events must be involved in the carcinogenic process.

Our present studies strongly support this hypothesis. We demonstrated that KBrO₃ treatment clearly induced DNA damage in both the alkaline and neutral COM assay (Fig. 1). The alkaline COM assay is capable of detecting any DNA damages including DSB, single strand breaks (SSB), alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites, while the neutral COM assay allows the detection of DSB, considered to be "biologically relevant" lesion of radiation damage [24]. KBrO₃ may have radio-mimic genotoxicity that yields oxidative DNA damage as well as DSB. KBrO₃ also induced MN formation and *TK* gene mutation significantly in TK6 cells. In the *TK* gene mutation assay, KBrO₃ predominantly produced SG mutants, but not NG mutants (Fig. 1c), implying that gross structural changes such as deletion and recombination are associated with the mutations. Molecular analysis of the *TK* mutants confirmed the assumption. Most of *TK* mutants showed LOH mutations, not non-LOH mutations, which are mainly point mutations. Harrington-Brock et al. also demonstrated that bromate compounds significantly induced *Tk* mutations in mouse lymphoma L5178Y cells, and almost all were LOH mutations [5]. LOH can be caused by deletions,

mitotic recombination between homologous alleles, or whole chromosome loss [20]. Molecular analysis can distinguish between them and reveal the mechanism and the characteristics of the mutants. In this study, KBrO_3 predominantly induced large deletions that resulted in hemizygous LOH (Table 1). The large deletions were mainly terminal deletions in the proximal region of chromosome 17q, which were rarely observed in spontaneously arising TK mutants (Fig. 3). The mutational spectrum and LOH pattern induced by KBrO_3 were similar to those induced by X-irradiation (Figs. 2 and 3) [20,21]. DSBs induced X-rays cause large deletions [19,20]. When the DSBs are repaired by the non-homologous end-joining pathway, interstitial deletions result. The broken chromosome ends can be also stabilized by the addition of new telomere sequences. Because TK6 cells have high telomerase activity [20], the result is terminal deletions. Thus, the major genotoxicity of KBrO_3 may be due to DSBs, but not to 8OHdG converting GC > TA transversion.

Some 8OHdG lesions can convert DSBs through the BER pathway [37]. In the initial step of BER, Ogg1 removes 8OHdG by DNA glycosylase activity and nicks the DNA backbone because of its associated lyase activity. The resulting SSB is processed by an apurinic endonuclease, which generates a single nucleotide gap. The gap is filled in by a DNA polymerase and sealed by a DNA ligase [38]. Clustered 8OHdG lesions induced by KBrO_3 may not be appropriately repaired by BER and cause DSB, however, because it is possible that two closely opposed 8OHdGs convert two closely opposed SSBs by BER resulting DSB [39,40]. Yang et al. developed Ogg1 over-expressing TK6 cell (TK6-hOGG1) and examined cytotoxic and mutagenic responses to gamma-irradiation [41]. They demonstrated that TK6-hOGG1 cells are more sensitive than the parental TK6 cells to cytotoxicity and mutagenicity by gamma-irradiation, and most of the induced TK mutants in TK6-OGG1 exhibited SG phenotype, which were probably large deletion mutants resulted by DSBs. This result clearly indicates that BER pathway contributes to convert oxidative damages to DSBs. Some clustered 8OHdG induced by KBrO_3 may convert to DSBs in TK6 cells, because TK6 is Ogg1 proficient cells [37].

To clarify the genotoxic characteristics of KBrO_3 , we investigated the gene expression profile using Affymetrix GeneChip[®] Expression analysis. Many genes were up- or down-regulated by exposure to 2.5 mM KBrO_3 (Tables 2 and 3). Akerman et al. investigated the alterations of gene expression profiles in ionizing radiation-exposed TK6 cells [42]. They reported a >50% increase in expression of ATF-3 (stress response), Cyclin

G (cell cycle), FAS antigen (apoptosis), GADD45 (repair and apoptosis), PCNA (repair), Rad51 (repair), and p21 (cell cycle) and a 40% decrease in expression of c-Myc (transcription factor), interferon stimulatory gene factor-3 (cell signaling), and p55CDC (cell cycle). We also observed up-regulation of p21 and down-regulation of c-Myc. Up-regulation of p21, however, is observed in TK6 cells exposed to any DNA-damaging chemical [43]. Islaih et al. also demonstrated the relationship between the gene expression profiles and the DNA damaging agents using TK6 cells [43]. They examined six chemicals including H_2O_2 and bleomycin which induce oxidative DNA damage. Although 10 genes were commonly up-regulated between H_2O_2 and bleomycin treatments, these genes except for p21 were not observed in our experiment. Thus, we could not find the similarity of gene expression profile by the treatment with KBrO_3 to by the treatment with ionizing radiation as well as oxidative damage inducers. Comparing gene expression profiles across platforms, laboratories, and experiments must be difficult [44]. Although it is difficult to judge from the expression analysis of the single chemical, information on genes which altered their expression gives a clue to understand the mechanism of action. Firstly, predominance of DNA repair and cell cycle related genes in up-regulated genes supports the genotoxic action of KBrO_3 . Up-regulation of stress genes and apoptosis related genes suggests an involvement of oxidative stress. Up-regulation of catalase may be responsible for the oxidative damage by KBrO_3 (Table 2). Unclassified genes for alteration may have a functional relationship with genotoxic mechanism.

In conclusion, KBrO_3 predominantly induced large deletions at chromosomal level in human TK6 cells. The major genotoxicity leading to carcinogenesis of KBrO_3 may be due to DSBs rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed.

Acknowledgments

The TK6 cell line used in this study was a kind gift of Dr. John B. Little of the Harvard School of Public Health, Boston, MA. This study was supported by Health, Welfare, and Labor Science Research Grants (H15-chem-002, H15-food-004) in Japan.

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A New Role of Thrombopoietin Enhancing *ex Vivo* Expansion of Endothelial Precursor Cells Derived from AC133-positive Cells*

Received for publication, May 14, 2007, and in revised form, September 5, 2007. Published, JBC Papers in Press, September 7, 2007, DOI 10.1074/jbc.M703919200

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We previously reported that CD31^{bright} cells, which were sorted from cultured AC133⁺ cells of adult peripheral blood cells, differentiated more efficiently into endothelial cells than CD31⁺ cells or CD31⁻ cells, suggesting that CD31^{bright} cells may be endothelial precursor cells. In this study, we found that CD31^{bright} cells have a strong ability to release cytokines. The mixture of vascular endothelial growth factor (VEGF), thrombopoietin (TPO), and stem cell factor stimulated *ex vivo* expansion of the total cell number from cultured AC133⁺ cells of adult peripheral blood cells and cord blood cells, resulting in incrementation of the adhesion cells, in which endothelial nitric oxide synthase and kinase insert domain-containing receptor were positive. Moreover, the mixture of VEGF and TPO increased the CD31^{bright} cell population when compared with VEGF alone or the mixture of VEGF and stem cell factor. These data suggest that TPO is an important growth factor that can promote endothelial precursor cells expansion *ex vivo*.

Neovascularization is an important adaptation to rescue tissue from critical ischemia. Postnatal blood vessel formation was formerly thought to be primarily due to the migration and proliferation of preexisting, fully differentiated endothelial cells, a process referred to as angiogenesis. Recent studies provide increasing evidence that circulating bone marrow-derived endothelial progenitor cells (EPCs)² contribute substantially to adult blood vessel formation (1–5). Cell therapy using EPCs is widely performed to rescue tissue damaged due to critical ischemia.

Although EPCs have been thought to be derived from many kinds of cells, cells characterized as CD34⁺ (6), AC133⁺ (7, 8),

* This work was supported in part by a grant-in-aid for health and labor science research from the Japanese Ministry of Health, Labor, and Welfare, and in part by a grant-in-aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: EPCs, endothelial precursor cells; VEGF, vascular endothelial growth factor; FN, fibronectin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TPO, thrombopoietin; SCF, stem cell factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; VEcad, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; STAT, signal transducers and activators of transcription; JAK, Janus kinase; KDR, kinase insert domain-containing receptor.

and CD14⁺ (9) are also thought to differentiate to EPCs. The main role of EPCs has been thought to be the release of angiogenic factors such as interleukin-8 (IL-8), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor, and vascular endothelial growth factor (VEGF) (9). To obtain a sufficient number of EPCs for the treatment may be very important in cell therapy for critical ischemia.

On the other hand, EPCs are mobilized from bone marrow by many substances such as G-CSF (10), granulocyte macrophage-colony stimulating factor (GM-CSF) (5), VEGF (3), erythropoietin (11–13), and statins (14, 15) *in vivo*. To get as many EPCs as possible without unduly burdening the patient, it is desirable to establish efficient expansion methods for EPCs *in vitro*.

Thrombopoietin (TPO), initially identified as the primary regulator of platelet production (16), plays an important and nonredundant role in the self-renewal of and expansion methods for hematopoietic stem cells (17–19). Recently, TPO has been found to exert a proangiogenic effect on cultured endothelial cells (20). The mechanism by which hematopoietic cytokines support revascularization *in vivo*, however, remains unknown. TPO has increased the number of colony-forming units-granulocyte-macrophage (21) and of burst-forming units-erythroid (22) *in vivo* and leads to a redistribution of colony-forming units-erythroid from marrow to spleen. Moreover, TPO acts in synergy with erythropoietin to increase the growth of burst-forming units-erythroid and the generation of colony-forming units-erythroid from marrow cells (21, 23, 24).

In our previous study (25), we isolated AC133⁺ cells and examined their endothelial differentiation *in vitro*. CD31(PECAM-1)⁺ and CD31^{bright} cells appeared at an early stage of the *in vitro* differentiation of AC133⁺ cells, and CD31^{bright} cells derived from AC133⁺ cells were identified as the precursors of endothelial cells because CD31^{bright} cells had differentiated more efficiently to endothelial cells than others. Therefore, we conclude that CD31^{bright} cells derived from AC133⁺ cells possess the typical character of EPCs. In this study, we analyzed the effects of TPO on the appearance of CD31^{bright} cells from AC133⁺ cells, and we show that TPO plays an important role in *in vitro* EPC expansion.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant TPO and recombinant stem cell factor (SCF) were kindly provided by Kirin-Amgen Inc. (Thousand Oaks, CA). Recombinant human VEGF was purchased from Strathmann Biotec AG (Hamburg, Germany). The AC133

Ex Vivo Expansion of EPC by TPO

magnetic cell sorting kit and phycoerythrin (PE)-conjugated anti-CD133/2 antibody were from Miltenyi Biotec (Gladbach, Germany). Allophycocyanin-conjugated anti-CD110 (TPO receptor) antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD31 monoclonal antibody, FITC-conjugated anti-CD34 monoclonal antibody, and anti-STAT3 monoclonal antibody were from Pharmingen. Phycoerythrin-conjugated vascular endothelial cadherin (VEcad/CD144) antibody was from Beckman Coulter (Marseilles, France). Anti-vascular endothelial growth factor receptor-2 (Flk-1/KDR) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-human endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibody (Cayman Chemical, Ann Arbor, MI) were obtained. Anti-phospho-Akt (Ser-473) antibody, anti-Akt antibody, and anti-phospho-STAT3 (Tyr-705) antibody were from Cell Signaling Technology (Beverly, MA). Fibronectin (FN)- and type IV collagen-coated dishes were purchased from Iwaki Co., Tokyo, Japan. Phycoerythrin-conjugated anti-CD14 antibody was from DakoCytomation (Glostrup, Denmark).

Preparation of Peripheral Blood Mononuclear Cells—Human cord blood was kindly supplied by the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) with informed consent. The buffy coat fraction was prepared from voluntary donated human blood of Saitama Red Cross of Japan (Saitama, Japan). The blood sample was diluted with phosphate-buffered saline (PBS) containing 2 mM EDTA and was loaded on a Lymphoprep™ tube (Axis-Shield PoC AS, Oslo Norway) (density = 1.077). After being centrifuged for 20 min $800 \times g$ at 18 °C, mononuclear cells were collected and washed with sorting solution (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin).

Flow Cytometric Analysis of AC133 and CD34 Expression in Mononuclear Cells—To eliminate the dead cells, dead cells were stained with 7-amino actinomycin D. Mononuclear cells were labeled with PE-conjugated anti-AC133 monoclonal antibody and FITC-conjugated anti-CD34 monoclonal antibody simultaneously at 4 °C for 30 min. After washing with the sorting solution, flow cytometric analysis was performed with a FACSCalibur (BD Biosciences).

Magnetic Cell Sorting of AC133⁺ Cells—Mononuclear cells were labeled with magnetic bead-conjugated anti-AC133 antibodies according to the protocol directed by the manufacturer. After the brief wash with the sorting solution, the cells were separated by a magnetic cell separator (autoMACS, Miltenyi Biotec, Gladbach, Germany), and the positive cells were then collected.

Culture of AC133⁺ Cells—Isolated AC133⁺ cells were cultured in EBM-2 (Cambrex Corp., East Rutherford, NJ) medium containing 20% heat-inactivated FBS and 30 mg/liter kanamycin sulfate at 37 °C under moisturized air containing 5% CO₂ with 50 ng/ml VEGF as control medium. Control medium containing VEGF was added with TPO, SCF, or both. Cells were plated on FN- or type IV collagen-coated dishes at a cell density of $\sim 10^6$ cells/ml. We have previously shown that EPCs can tightly adhere to an FN-coated dish but weakly to type IV collagen-coated dish (25). Analysis of adherent EPCs was performed on FN-coated dish and that of suspended EPCs on type IV collagen-coated dish. Half of the medium was exchanged

once every 3–4 days with fresh medium. Adherent cells on FN-coated dish were fixed with ethanol chilled to -20 °C and then subsequently subjected to an immunostaining procedure or other treatments. Cells on type IV collagen-coated dish were subsequently subjected to flow cytometric analysis.

Immunostaining of Adherent Cells—After fixation with chilled ethanol (-20 °C), the cell layer was washed three times with PBS. Cells were incubated with 1% bovine serum albumin in PBS (–) for 1 h at 4 °C for blocking and then with each first antibody in 1% bovine serum albumin in PBS (–) for 1 h at 4 °C. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse IgG antibody or rhodamine-conjugated anti-rabbit IgG antibody for 1 h at 4 °C. Cells were washed with PBS and then examined using a Zeiss LSM 510 microscope with an excitation wavelength of 488 nm and an emission of 530/30 nm for FITC or 570/30 nm for rhodamine.

In every experiment, we used nonspecific immunoglobulin corresponding to the first antibody species as a control and confirmed that the cells were not stained with control immunoglobulin. The fluorescence intensity of 20 randomly selected cells was calculated using the Scion Image program within the linear range for quantitation.

Analysis of Cytokines in the Supernatant of CD31^{bright} and CD31⁺ Cells—The expression of CD31 on cultured AC133⁺ cells was determined with a flow cytometer. After AC133⁺ cells were cultured for several days on either FN-coated or collagen type IV-coated dishes, both adherent and nonadherent cells were collected. The collected cells were labeled with FITC-labeled anti-CD31 antibody for 15 min at 4 °C. After a brief wash with 0.5% bovine serum albumin in PBS, flow cytometric analysis was performed. CD31^{bright} and CD31⁺ cells were sorted from cultured AC133⁺ cells with FACSAria (BD Biosciences). Sorted cells of both populations were subsequently cultured in EBM-2 supplemented with 20% FBS in the absence of any cytokines. After 5 days, the collected supernatant of cells was frozen at -20 °C. Cytokines were measured by a BD™ cytometric beads array Flex set system (BD Biosciences) according to the manufacturer's protocol.

Flow Cytometric Analysis of Various Cell Surface Markers in Cultured AC133⁺ Cells—After AC133⁺ cells were cultured for the indicated period, cells were co-stained with FITC-labeled anti-CD31 antibody and PE-labeled anti-CD14 antibody or PE-labeled VEcad antibody. Cells were also stained with FITC-labeled anti-CD31 antibody, allophycocyanin-labeled anti-CD110 antibody, and PE-labeled anti-AC133 antibody triply and then subjected to flow cytometry. Dead cells were eliminated by staining with 7-amino actinomycin D.

Calculation of the Absolute Number of CD31^{bright} Cells—The absolute number of CD31^{bright} cells was multiplied by the total cell number of each well, and the ratio of CD31^{bright} cells was analyzed by fluorescence-activated cell sorter.

Preparation of Cell Lysates and Immunoblotting—After cell sorting, AC133⁺ cells were suspended in 20% FBS-EBM2 and cultured for 3 days in the presence of VEGF and TPO. Cells were collected and incubated in 2% FBS-EBM2 for 1 h. Cells were stimulated by 50 ng/ml TPO, 50 ng/ml VEGF, or both for 15 min. Cells (1×10^6) were collected and lysed in lysis buffer containing 1% Triton X-100, 10 mM K₂HPO₄/KH₂PO₄ (pH

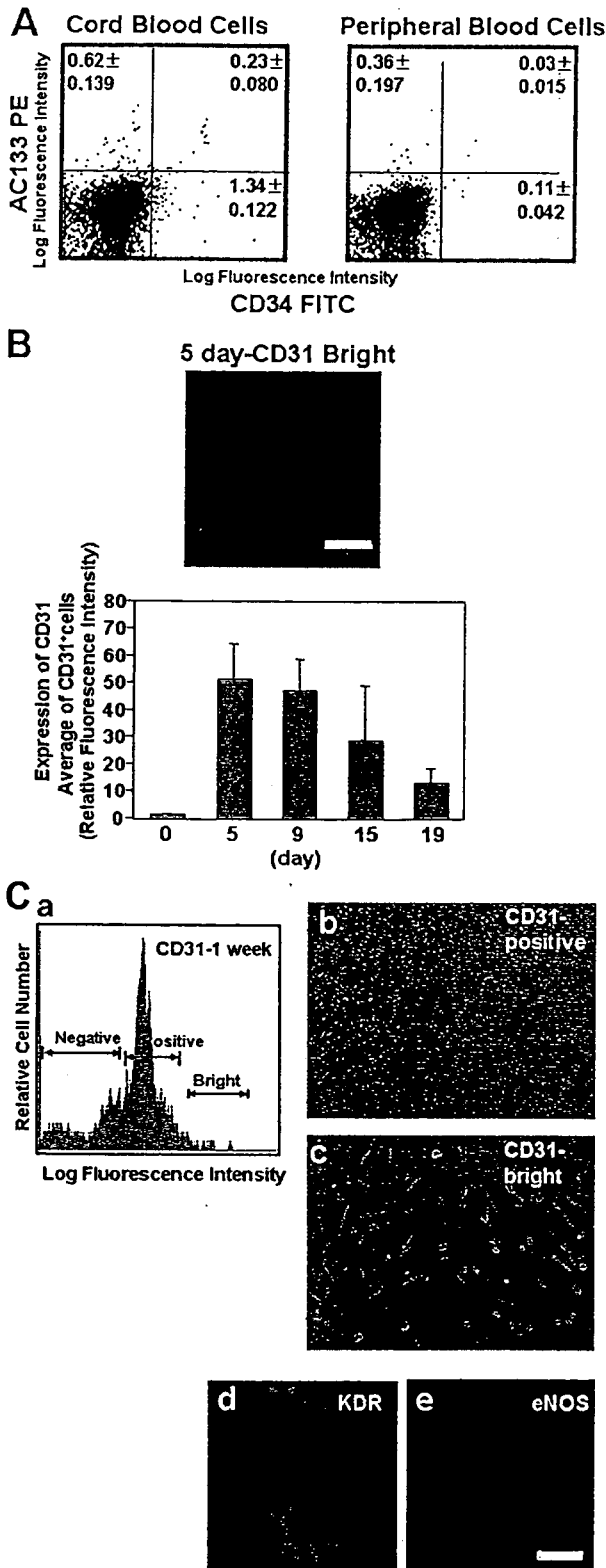


FIGURE 1. *In vitro* differentiation of AC133⁺ cells of cord blood into endothelial cells. **A**, expression of AC133 and CD34 cells in human cord blood and peripheral blood mononuclear cells was analyzed by staining with AC133-PE (vertical axis) and CD34-FITC (horizontal axis). The numbers in the flow cytometric dot plots indicates the percentage of each population ± S.D. **B**, when AC133⁺ cells were cultured for 19 days in the presence of VEGF on FN-coated dishes, the appearance of CD31⁺ cells was analyzed. The upper panel shows the fluorescent photomicrograph of adhesion cells stained with FITC-conjugated

7.5), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl₂, and 50 mM β-glycerophosphate, along with 1/100 (v/v) protease inhibitor mixture (Sigma) and 1/100 (v/v) phosphatase inhibitor mixture (Sigma). The cellular lysate of 5 × 10⁵ cells/lane was subjected to Western blotting analysis.

Statistical Analysis—Statistical analysis was performed using the unpaired Student's *t* test, and the dose response of TPO was compared between the four groups by one-way analysis of variance and the Tukey test using Prism 4 software. Values of *p* < 0.05 were considered to indicate statistical significance. Each experiment was repeated three times, and the representative data are indicated.

RESULTS

We previously reported that during the *in vitro* differentiation of peripheral blood AC133⁺ cells into the endothelial cells, the expression of CD31 was the earliest marker among all of the tested markers (25). Moreover, by analyzing the ability of differentiation into endothelial cells, CD31^{bright} cells were shown to exhibit EPC character when compared with the CD31⁺ fraction. Since cord blood is a rich source of blood stem cells such as CD34⁺ and AC133⁺ cells, it is expected to be a useful source for CD31^{bright} cells. At first, we attempted to determine whether the CD31^{bright} fraction derived from cord blood AC133⁺ cells contained EPCs. As shown in Fig. 1A, the populations of AC133⁺ CD34⁻ cells, AC133⁻ CD34⁺ cells, and AC133⁺ CD34⁺ cells in cord blood were approximately four times greater than those in peripheral blood (Fig. 1A). After 5 days of cultivation of AC133⁺ cells on an FN-coated dish, adherent CD31-positive cells were observed (Fig. 1B, upper panel). Analysis of the fluorescence intensity of CD31-positive cells revealed that the average fluorescence intensity in CD31⁺ cells was highest on day 5 (Fig. 1B, lower panel), corresponding to the results of peripheral blood cells.

After 1 week of cultivation of AC133⁺ cells on a collagen type IV-coated dish, on which cells adhered more loosely when compared with the FN-coated dish, cells were collected and sorted into CD31⁺ and CD31^{bright} fractions, as shown in Fig. 1C, panel a, and both cell types were cultured on an FN-coated dish for 1 week after the sorting. The number of cells adhering and spreading was higher in the CD31^{bright} fraction (Fig. 1C, panel c) than in the CD31⁺ fraction (Fig. 1C, panel b), and these adhering cells are apparently KDR- (Fig. 1C, panel d) and eNOS-positive (Fig. 1C, panel e). The large areas of intense green fluorescence represent the colonies of CD31^{bright} cells. These data indicate that CD31^{bright} cells derived from AC133⁺ cells of both peripheral blood and cord blood are EPCs.

anti-CD31 antibody after a 5-day culture. Quantitation of the fluorescence intensity of 20 CD31-positive cells was analyzed as described under "Experimental Procedures." Columns and bars represent the means ± S.D. from 20 cells (B, lower panel). C, the CD31-negative, positive, and bright cell populations prepared after 1-week cultivation of AC133⁺ cells are shown in a representative histogram stained with FITC-conjugated anti-CD31 antibody. The x axis represents the log fluorescence intensity of CD31-FITC, y axis relative cell number (panel a). Panels b and c show phase-contrast microscopic photographs of cultured CD31-positive and bright cells, respectively, subsequently cultured for 1 week after cell sorting. The bottom panels d and e show the fluorescent photomicrographs of adhesion cells from the CD31^{bright} fraction stained with anti-KDR antibody and anti-eNOS antibody, respectively. Scale bar, 100 μm.

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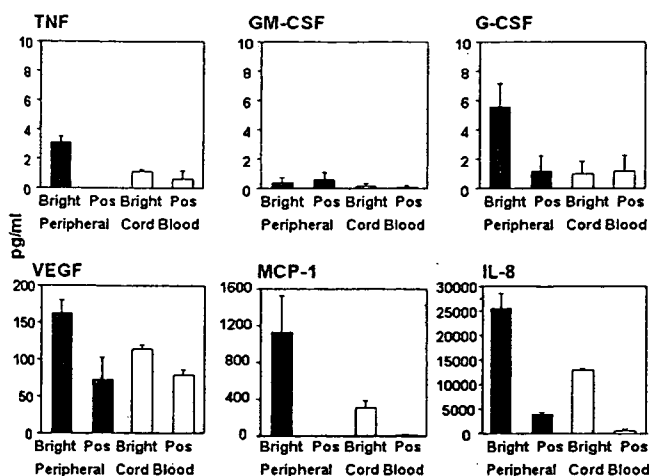


FIGURE 2. Various cytokines released from CD31⁺ cells and CD31^{bright} cells. Production of various cytokines from CD31⁺ cells and CD31^{bright} cells derived from AC133⁺ cells cultivated for 5 days was measured. Gray columns indicate the cytokine production by cells from peripheral blood and open columns from cord blood. Columns and bars represent the means \pm S.D. from three separate experiments. *TNF*, tumor necrosis factor; *Pos*, positive; *MCP-1*, monocyte chemoattractant protein-1.

Several reports have shown that EPCs produce cytokines (9, 26, 27), but the ability of CD31⁺ or CD31^{bright} cells derived from AC133⁺ cells to produce cytokines is not known. After cell sorting, quantitative analysis of cytokines released by CD31⁺ cells and CD31^{bright} cells was carried out at 5 days after the cultivation. As shown in Fig. 2, IL-8 was markedly produced by CD31^{bright} cells from both peripheral blood and cord blood when compared with CD31⁺ cells. The production of monocyte chemoattractant protein-1 (MCP-1) by CD31^{bright} cells was also higher than that of CD31⁺ cells. The production of VEGF was higher by CD31^{bright} cells than by CD31⁺ cells but not significantly. The production of all cytokines by CD31^{bright} cells from peripheral blood was higher than that from cord blood. Tumor necrosis factor- α , GM-CSF, and G-CSF were hardly produced by CD31^{bright} and CD31⁺ cells. These data indicate that CD31^{bright} cells derived from AC133⁺ cells have a strong ability to produce chemokines.

It has been reported that TPO and SCF are potent stimulators of multipotent cell proliferation (17, 19). Next, the effects of both growth factors on EPC growth and differentiation in our culture system were determined. After the addition of both TPO and SCF for 2 weeks, the expression of eNOS and KDR in adhered cells was analyzed (Fig. 3A). Fig. 3A clearly indicates that AC133⁺ cells from both peripheral blood and cord blood differentiate into eNOS⁺ and KDR⁺ cells more efficiently in the presence of the mixture of TPO, SCF, and VEGF than of VEGF alone. Flow cytometric analysis revealed that the ratio of CD31^{bright} CD14⁻ cells increased in the presence of the mixture of TPO, SCF, and VEGF when AC133⁺ cells were cultured on collagen type IV-coated dish for 1 week (Fig. 3B).

We next examined which growth factor is dominant in the induction and proliferation of CD31^{bright} cells. The total cell number of cultured AC133⁺ cells from both peripheral blood (Fig. 4A, upper panel) and cord blood (Fig. 4A, lower panel) significantly increased in the presence of TPO, SCF, or both growth factors when compared with that of VEGF alone during

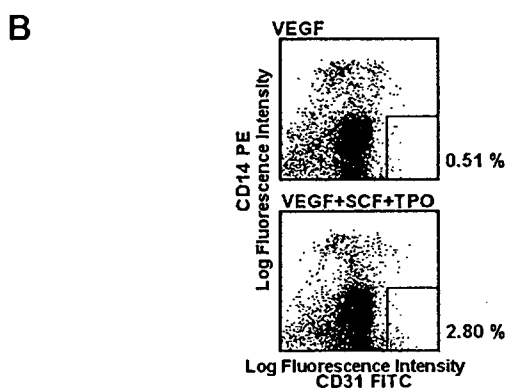
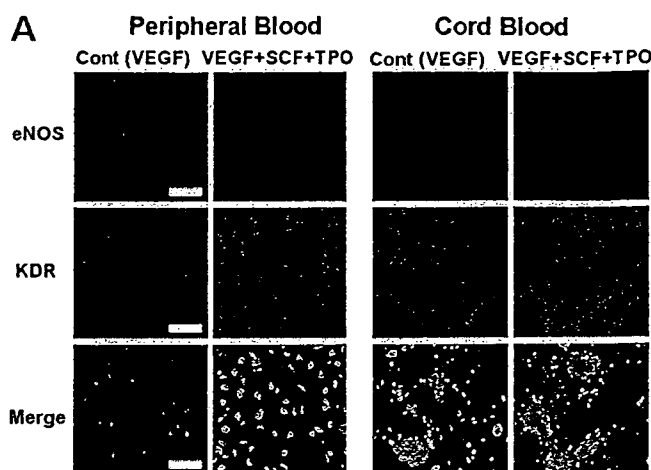


FIGURE 3. Increment of EPCs from AC133⁺ cells in the presence of TPO and SCF. A, AC133⁺ cells were differentiated for 2 weeks in the presence of either VEGF alone or the combination of TPO, SCF, and VEGF on an FN-coated dish. The upper and middle panels indicate the fluorescent photomicrographs of cells stained with anti-eNOS antibody and anti-KDR antibody, respectively. The bottom panels indicate the merged images of both antibodies. From the left side, control (Cont) and the mixture of peripheral blood, control, and the mixture of cord blood. Scale bar, 100 μ m. B, CD14 and CD31 expression in cultured AC133⁺ cells for 1 week was stained with CD14-PE (vertical axis) and CD31-FITC (horizontal axis). The upper panel indicates cells treated with VEGF alone, and the lower panel indicates cells treated with the mixture of VEGF, SCF, and TPO. The number on the right side of the flow cytometric dot plot indicates the percentage of the CD14⁻ CD31^{bright} population.

a 1-week period. As shown in Fig. 4B, however, the increment in the ratio of the CD31^{bright} cell population was observed only in the presence of TPO. The absolute number of CD31^{bright} cells, calculated by the total cell number and the ratio of the CD31^{bright} cell population, was markedly increased by TPO (Fig. 4C). In contrast, SCF induced the increase in total cell number to the same level as TPO (Fig. 4A), but it did not induce the increase in either the ratio of the CD31^{bright} cell population (Fig. 4B) or the number of CD31^{bright} cells (Fig. 4C). Next, we examined whether TPO and VEGF can synergistically affect the induction of CD31^{bright} cells during a 1-week cultivation. As shown in Fig. 4D, although VEGF had no effects on the total cell number (Fig. 4D, panel a), it increased the ratio of the CD31^{bright} cell population to 1.4-fold higher than that of the control (Fig. 4D, panel b), resulting in a slight increase in the number of CD31^{bright} cells (Fig. 4D, panel c). Thrombopoietin alone induced an increase in not only the total cell number (Fig. 4D, panel a) but also the ratio of the CD31^{bright} cell population (Fig. 4D, panel b), resulting in an \sim 24-fold increment of the absolute

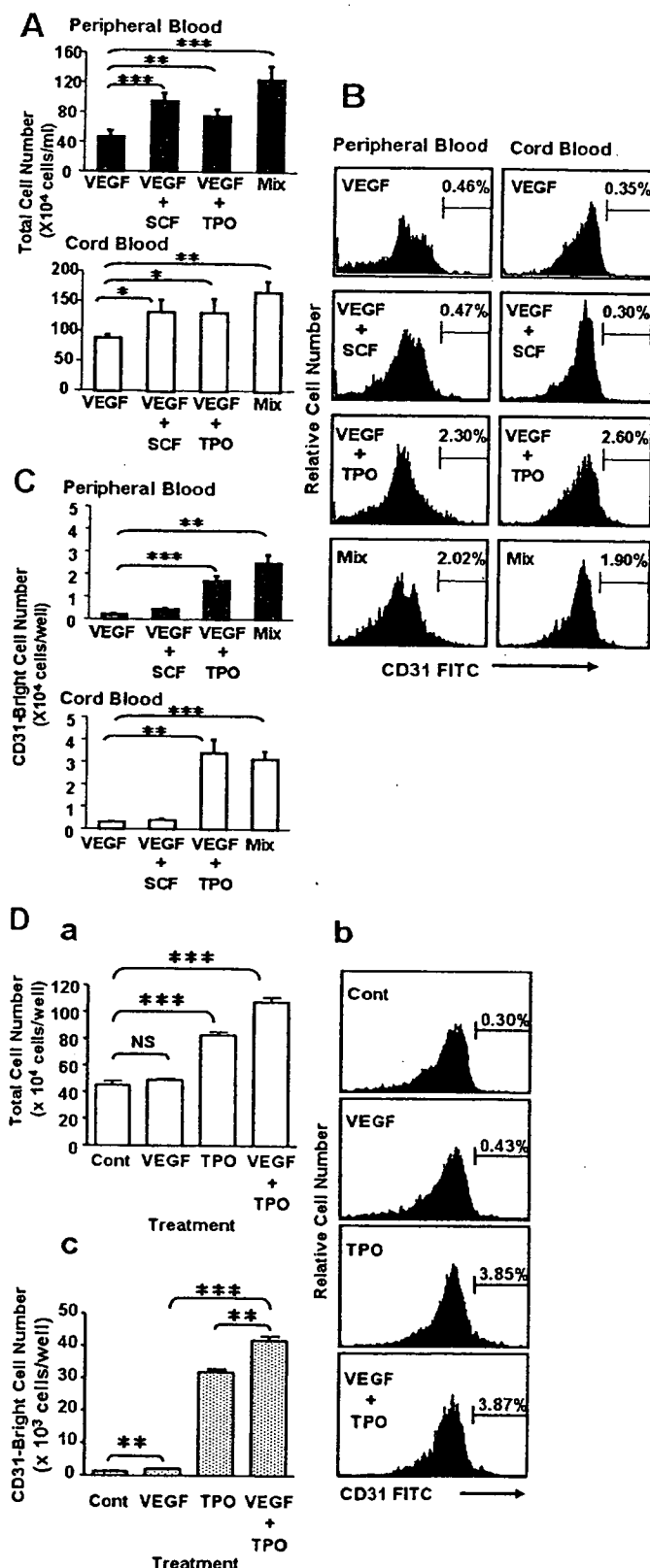


FIGURE 4. Stimulative effects of TPO on induction of CD31^{bright} cells. A, alteration of the cell number of cultivated AC133⁺ cells for 1 week in the combination of growth factors. Mix, VEGF + SCF + TPO. B, the flow cytometric histogram of AC133⁺-derived cells stained with FITC-labeled anti-CD31 antibody after a 1-week culture. The representing number in the flow cytometric histogram indicates the percentage of the CD31^{bright} cell population. The left panels are peripheral blood, and the right panels are cord blood. C, CD31^{bright}

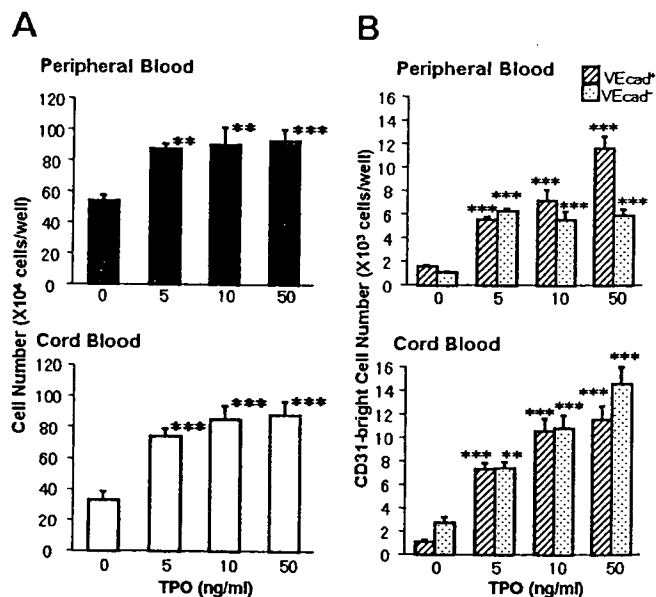


FIGURE 5. Dose-dependent effects of TPO on the induction of CD31^{bright} cells from AC133⁺ cells. AC133⁺ cells were treated with various concentrations of TPO for 1 week. The left panels (A) are the total cell number of cultured AC133⁺ cells from peripheral blood (upper panel) and cord blood (lower panel). The right panels (B) are the calculated CD31^{bright} cell number from peripheral blood (upper panel) and cord blood (lower panel). Columns and bars represent the means \pm S.D. (**, $p < 0.01$; ***, $p < 0.001$). Striped and dotted columns represent CD31^{bright}VEGad⁺ cells and CD31^{bright}VEGad⁻ cells, respectively.

number of CD31^{bright} cells when compared with the control (Fig. 4D, panel c). The concomitant treatment with both VEGF and TPO showed a synergic increase in the number of CD31^{bright} cells (Fig. 4D, panel c).

When AC133⁺ cells were cultured with various concentrations of TPO in the presence of constant concentrations of VEGF (50 ng/ml), the total cell number from both peripheral blood (Fig. 5A, upper panel) and cord blood (Fig. 5A, lower panel) significantly increased at 5 ng/ml of TPO when compared with the control, and there was no significant difference in the total cell number from 5 to 50 ng/ml of TPO. However, TPO increased the ratio of CD31^{bright} cells of flow cytometry dose-dependently as follows: control, 0.50%; 5 ng/ml, 1.36%; 10 ng, 1.42%; 50 ng/ml 1.90% in peripheral blood and control, 1.16%; 5 ng/ml, 1.99%; 10 ng, 2.51%; 50 ng/ml 2.96% in cord blood. TPO markedly induced the differentiation of AC133⁺ cells into CD31^{bright}VEGad⁺ cells in the case of both peripheral blood (Fig. 5B, upper panel) and cord blood (Fig. 5B, lower panel) in a dose-dependent manner. In the case of cord blood cells, differentiation into CD31^{bright}VEGad⁻ cells was also induced by TPO.

The effects of TPO on total cell number during 6-day culture of AC133⁺ cells were determined. Although the total cell num-

bers were calculated by both the total cell number and the ratio of CD31^{bright} population. D, the effects of TPO alone on EPC differentiation derived from AC133⁺ cells of cord blood. The upper left panel (a) shows the total cell number after a 1-week culture, the right panels (b) show the flow cytometric histogram of AC133⁺-derived cells stained with FITC-labeled anti-CD31 antibody, and the lower left panel (c) shows the calculated CD31^{bright} cell number. Columns and bars represent the means \pm S.D. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). NS, not significant; Cont, control.

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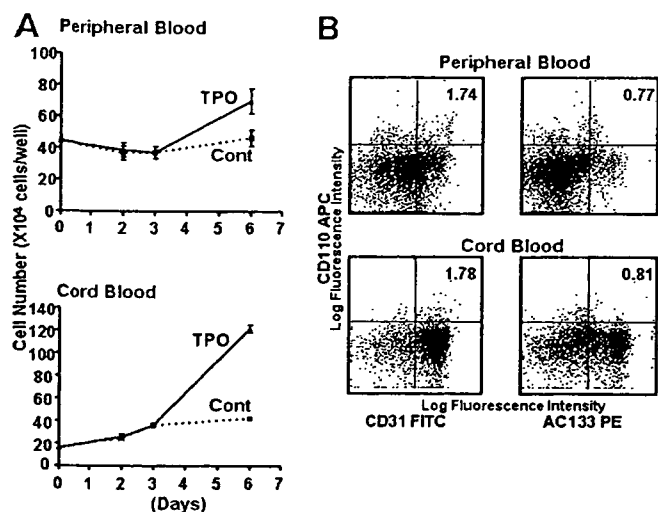


FIGURE 6. Time-course analysis of TPO-treated AC133⁺ cells and expression of TPO receptor (CD110). *A*, alteration of cell number was counted at 2, 3, and 6 days. *Solid* and *dotted* lines indicate TPO-treated cells and control ((*Cont*) VEGF alone) cells, respectively. The results represent mean \pm S.E. of triplicate wells. *B*, flow cytometric analysis of CD110 expression on AC133⁺ cells cultured for 3 days was carried out. The *y* axis represents the log fluorescence intensity of CD110-allophycocyanin (APC), and the *x* axis represents that of CD31-FITC (*left panels*) and AC133-PE (*right panels*). The number in the flow cytometric dot plot indicates the percentage of CD110⁺ CD31⁺ and CD110⁺ AC133⁺ populations, respectively. The *upper panels* are peripheral blood, and the *lower panels* are cord blood.

ber from AC133⁺ cells slightly and constantly increased from day 0 to day 6 in the absence of TPO, total cells markedly increased after the third day in the presence of TPO (Fig. 6A). Next, the alternation of TPO receptor (CD110) expression was analyzed during the cultivation of AC133⁺ cells. Although the percentages of both AC133⁺ CD110⁺ cells and CD31⁺ CD110⁺ cells were 0% just after magnetic cell sorting, 3 days after the cultivation, ~2% of CD31⁺ CD110⁺ cells (Fig. 6B, *left panel*) and 1% of AC133⁺ CD110⁺ cells (Fig. 6B, *right panel*) appeared from AC133⁺ cells in the peripheral blood and cord blood, respectively. These data indicate the possibility that sorted AC133⁺ cells may differentiate into AC133⁺ CD110⁺ cells and may subsequently proliferate and differentiate into EPCs in response to TPO.

It has been reported that TPO activates the PI3K/Akt pathway (28) or JAK/STAT pathway (20, 29, 30) in target cells. In addition, in the present study, TPO induced a marked proliferation of AC133⁺ cells after 3-day culture, and CD110 expression in cells cultured for 3 days from both cord blood and peripheral blood was also observed (Fig. 6, *A* and *B*). We then attempted to determine whether TPO activates Akt or STAT in AC133⁺ cells cultured for 3 days by analyzing the phosphorylation at Ser-473 of Akt or the phosphorylation at Tyr-705 of STAT3, which are the active forms of Akt or STAT3, respectively. As shown in Fig. 7A, phosphorylation at Ser-473 of Akt was stimulated by both VEGF and TPO at 15 min and was more markedly stimulated by concomitant treatment with VEGF and TPO than by a single treatment (Fig. 7A, *top panel*). Phosphorylation at Tyr-705 of STAT3 was observed only in the presence of TPO, and unlike in the phosphorylation at Ser-473 of Akt, an increased amount of phosphorylation was not observed in the concomitant presence of VEGF and TPO (Fig. 7A, *third panel*).

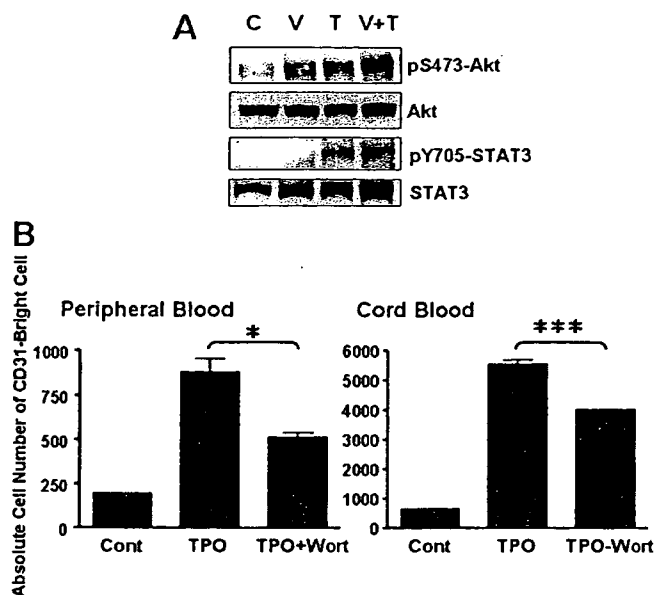


FIGURE 7. Analysis of TPO-induced signal transduction on AC133⁺ cells of cord blood. *A*, activation of Akt or STAT3 was analyzed by Western blotting with anti-phospho-specific Ser-473-Akt antibody (*top panel*) and reprobed with anti-Akt antibody (*second panel*), or with anti-phospho-specific Tyr-705-STAT3 antibody (*third panel*) and reprobed with anti-STAT3 antibody (*lower panel*) after stimulation by VEGF, TPO, or both VEGF and TPO for 15 min using 3-day-cultured AC133⁺ cells. *C*, control; *V*, VEGF; *T*, TPO. *B*, the effects of wortmannin on CD31^{bright} cell induction were investigated. The *right panel* shows peripheral blood, and the *left panel* shows cord blood. The *y* axis represents the CD31^{bright} cell number. *Wort*, 100 nM wortmannin. Columns and bars represent the means \pm S.E. (*, $p < 0.05$; ***, $p < 0.001$). *Cont*, control.

On the other hand, there was no difference in the expression of Akt and STAT3 protein levels (Fig. 7A, *second panel* and *bottom panel*, respectively). The induction of CD31^{bright} cells was not perfectly but significantly inhibited by wortmannin, an inhibitor of PI3K, suggesting that the PI3K/Akt pathway plays an important role in TPO-induced EPC differentiation (Fig. 7B).

DISCUSSION

We have previously reported that CD31^{bright} cells derived from AC133⁺ cells in human peripheral blood are EPCs (25). In the present study, CD31^{bright} cells also appeared from AC133⁺ cells prepared from cord blood, which are a rich source of stem cells during the early period of cultivation (Fig. 1, *A* and *B*). When cells were separated in terms of CD31 expression (Fig. 1C), CD31^{bright} cells differentiated into KDR-positive and eNOS-positive adherent cells. These data indicate that CD31^{bright} cells derived from AC133⁺ cells in cord blood have some characteristics similar to those of EPCs in peripheral blood. Although these EPCs in both cord blood and peripheral blood could not form tube-like structure by themselves on Matrigel (data not shown), they secreted angiogenic growth factors (Fig. 2) such as VEGF, IL-8 (31, 32), and monocyte chemoattractant protein-1 (MCP-1) (33). It has been reported that there are at least two types of EPCs: early EPCs and late EPCs. Early EPCs are unable to form tube-like structures and secrete VEGF and IL-8 showing peak growth at 2–3 weeks (9, 26, 27). Late EPCs with the ability to proliferate and having a cobblestone shape appear late at 2–3 weeks, show exponential growth at 4–8 weeks, and have the ability to form tube-like structures

(26, 27, 34). Rehman *et al.* (9) have reported that EPCs derived from monocytes/macrophages do not proliferate but instead release potent proangiogenic growth factors. In many studies (9, 26, 27, 35–37), because the origin of early EPCs was CD14⁺ cells or was not precluded by monocytic cells, CD14 expression was still observed in the EPCs after cultivation. In our study, in which AC133⁺ cells were used as the origin of the EPCs, CD14 expression was not observed in CD31^{bright} cells induced by TPO (Fig. 3B). Although the CD31^{bright} cells identified as EPCs in this report and in a previous report did not correspond to their cells in terms of the origin of the cells or cell surface markers, these cells may be early EPCs that can release potent proangiogenic growth factors (Fig. 2). In any event, EPCs are thought to be a heterogeneous population, unlike late EPCs, which have a high ability to proliferate.

Circulating EPCs are up-regulated under physiological or pathological conditions and also by 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (14, 15) and cytokines such as erythropoietin (11–13) and G-CSF (10). In this report, we have revealed the possibility of marked expansion of EPCs *in vitro* by TPO. Brizzi *et al.* (20) have reported that TPO directly stimulates endothelial cell motility and neoangiogenesis. In the present study, TPO may have played a stimulatory role in the differentiation of EPCs from circulating stem cells.

Although both TPO and SCF have the same potency with regard to proliferation of AC133⁺ cells (Fig. 4A), TPO specifically induces an increase in the ratio of the CD31^{bright} cell population when compared with SCF (Fig. 4, B and C). To develop useful cell therapy products for severe ischemia, it has been considered desirable to establish the efficient expansion of EPCs *in vitro*. Thrombopoietin could increase CD31^{bright} cells (EPCs) even in the absence of VEGF. Kirito *et al.* (38) have reported that TPO enhances expression of VEGF in hematopoietic cells through induction of hypoxia-inducible factor 1 α . These observations suggest the possibility that the production of EPCs by TPO may be supported by VEGF produced by AC133⁺ cells. However, from the perspective that TPO and VEGF have synergistic effects on the induction of EPCs, TPO seems to induce EPCs through another signaling cascade.

Thrombopoietin is a major regulator of the proliferation, differentiation, and maturation of megakaryocytes (39, 40). The results from recent studies suggest that TPO can act not only as a lineage-specific hematopoietic growth factor but also can affect other hematopoietic cell types. For example, TPO alone does not induce proliferation of long term repopulating hematopoietic stem cells. However, in combination with SCF or IL-3, TPO has several synergistic effects on cell proliferation (19). Our results have revealed a new role of TPO in the production of EPCs.

In the process of differentiation of AC133⁺ cells into CD31^{bright} cells, both peripheral blood and cord blood appear to be very similar. AC133⁺ cells of cord blood, however, have a stronger ability to proliferate than those of peripheral blood (Fig. 6A). Moreover, TPO stimulates the induction of CD31^{bright}VEcad⁻ cells only from cord blood (Fig. 5B) at high concentrations. Hur *et al.* (26) have reported that VEcad⁻ EPCs are thought to be an early EPC. It is therefore thought that AC133⁺ cells of cord blood are more immature than those of peripheral blood.

Although the total cell number treated with TPO slightly increased in a dose-dependent manner (Fig. 5A), the CD31^{bright} cell number markedly increased as the TPO concentration increased (Fig. 5B). These data suggest the possibility that a higher concentration of TPO may be needed for CD31^{bright} cell induction from AC133⁺ cells.

When AC133⁺ cells were stimulated by TPO or VEGF, an increase in the phosphorylation of Akt at Ser-473 was observed. This increase was strongly enhanced by concomitant treatment with VEGF and TPO (Fig. 7A). The induction of CD31^{bright} cells by these growth factors (Fig. 4D) was consistent with the increase in the phosphorylation of Akt at Ser-473. TPO but not VEGF could also stimulate the phosphorylation of STAT3 at Tyr-705. We previously reported that the PI3K/p70 S6 kinase pathway and the JAK/STAT3 pathway were important for proliferation and differentiation, respectively, in neutrophilic differentiation (41, 42). Owing to the stimulation of both the PI3K/Akt and the JAK/STAT pathways, we postulated that TPO may be a stronger stimulator of EPC production than VEGF. As shown in Fig. 7B, however, wortmannin could not completely inhibit the induction of CD31^{bright} cells. Therefore, a pathway other than the PI3K/Akt pathway may also work for the proliferation and differentiation of EPCs.

The observation of unfavorable angiogenesis has recently been reported after transplantation of bone marrow mononuclear cells in patients with thromboangiitis obliterans (43). Moreover, transfer of both spleen cell-derived EPCs and bone marrow mononuclear cells accelerate atherosclerosis in apoE knockout mice, whereas EPC transfer reduces markers associated with plaque stability (44). These observations suggest that transplantation of differentiated cells from EPCs may be useful therapy as regenerative medicine.

In conclusion, we have demonstrated a new role of TPO in enhancing the differentiation of AC133⁺ cells into CD31^{bright} cells (EPCs) *in vitro*. These findings may contribute to further development of cell therapy for critical ischemia.

Acknowledgments—We thank Saitama Red Cross of Japan (Saitama, Japan) and Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) for their kind cooperation. We also thank Kirin-Amgen Inc. for their kind gift of recombinant TPO and recombinant SCF.

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