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Conditioned media of glial cell lines induce alkaline phosphatase activity in cultured artery endothelial cells

Identification of interleukin-6 as an induction factor

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Abstract

Conditioned media of human glial cell lines induced alkaline phosphatase activity in cultured calf artery endothelial cells. The maximal alkaline phosphatase activity in the culture was comparable to the level in isolated brain capillary endothelial cells. An induction factor in the conditioned media was purified and identified as interleukin-6 from its amino-terminal sequence, molecular weight, amino acid composition and immunoreactivity. Recombinant interleukin-6 had similar induction activity. Our findings raise the possibility that interleukin-6 induces and modulates alkaline phosphatase activity in endothelial cells during normal development of the blood–brain barrier and under certain pathological conditions.

Key words: Alkaline phosphatase; Endothelial cell; Glioma; Blood–brain barrier; Interleukin-6; Differentiation

1. Introduction

Brain capillary endothelial cells, site of the blood–brain barrier (BBB), are specifically differentiated; they are impermeable to most blood-borne molecules and express many kinds of transporters and enzymes for uptake of nutrient molecules [1–3]. In the brain, alkaline phosphatase (ALP; EC 3.1.3.1) activity is markedly high in endothelial cells of BBB-type microvessels, and negative or low in those of non-BBB type vessels [1,2,4,5]. The ALP levels parallel the maturation of BBB in the newly-formed capillary endothelial cells during normal development in the brain [2,4,6] and during repair processes after brain injury and inflammation [7]. Thus, ALP has been accepted as a marker enzyme for the BBB [1–4].

Stewart and Wiley [8] showed that abdominal vessel vascularizing grafted neural tissue became impermeable to circulating dye and expressed high ALP activity. They suggested that BBB properties, i.e. restricted permeability and expression of many BBB marker proteins, are not natural endowments of the endothelium of the BBB, but can be induced, even in endothelial cells of peripheral tissue origin. The nervous cells surrounding the brain capillary endothelial cells, i.e. astrocytes, pericytes, and/or neurons, are likely to be responsible for the induction of differentiated BBB endothelial cells [1–3]. Conflicting studies have been reported for the induction mechanism, whether cell-to-cell contact or soluble factors are needed [1–3,9–16]. No molecule derived from these cells has yet been identified which induces any BBB markers in endothelial cells.

In this study, we focused on conditioned media (CM) of various human glial cell lines and examined them for their ability to induce ALP activity in cultured endothelial cells. Our preliminary studies using a rapid, sensitive screening method for detecting ALP activity in cultured cells [17] showed that ALP activity was elevated in calf pulmonary artery endothelial (CPAE) cells cultured with CMs of human glial cell lines; especially high activity was noted for CM of human glioma Hs683 cells. A factor that elevated the ALP activity was purified from Hs683 CM, and identified as interleukin-6 (IL-6). Possible biological implications of our findings are discussed.

2. Experimental

2.1. Recombinant IL-6 and antibodies

Recombinant human IL-6 (rIL-6), purified from CM of Chinese hamster ovary cells, and mouse monoclonal antibody to this rIL-6 preparation were from Genzyme Corp. (MA, USA). The rIL-6 was used after its actual content in the material was determined by an immunoassay as stated below (section 2.9). Mouse anti-IL-6 monoclonal antibody (B-E8) was from Boehringer-Mannheim Biochemic (IN, USA).

2.2. Cell culture

Human cell lines, Hs683 (glioma, ATCC No. HTB 138), H4 (neuroglioma, ATCC No. HTB 148), A172 (glioblastoma, ATCC No. CRL 1620), U-373 MG (glioblastoma, ATCC No. HTB 17), T98G (glioblas-

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Abbreviations: BBB, blood–brain barrier; ALP, alkaline phosphatase; CM, conditioned media; CPAE cells, calf pulmonary artery endothelial cells; IL-6, interleukin-6; rIL-6, recombinant human IL-6; FCS, fetal calf serum; DOC, deoxycholate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay.

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toma, ATCC No. CRL 1690), IMR-32 (neuroblastoma ATCC No. CCL127), KD (lip fibroblast, ATCC No. CRL 1295), and KHOS-240S (osteosarcoma, ATCC No. CRL 1545), were cultured in collagen-coated dishes (Corning 100 mm in diameter) in DMEM (Dulbecco's modified Eagle medium containing 10% fetal calf serum (FCS)). CPAE (calf pulmonary artery endothelial cells) (ATCC No. CCL209) were cultured in EMEM (Eagle's minimum essential medium containing 20% FCS). On induction experiments, CPAE cells were cultured for 4 days or longer with a 1:1 mixture of the EMEM and the sample medium (CM, the DMEM or chromatography fraction diluted with the DMEM). HUVEC-C (umbilical cord endothelial cells ATCC No. CRL 1730) were cultured in collagen-coated dishes in 199 media with 20% FCS supplemented with nutrients as described before [18]. PASMC (human pulmonary aortic smooth muscle cells, Sanko Junyaku, Osaka, Japan) were cultured according to the supplier's manual.

2.3. Preparation of CM

Hs683 cells were seeded in collagen-coated dishes (10^6 cells/dish in 10 ml of the DMEM). Four days later, the medium of confluent culture was replaced with a fresh one and 48 h later, the CM was harvested and filtrated with a sterile 0.2 μm filter and stored frozen at −20°C until use. In CMs of other cell lines, the media of confluent culture were replaced with 10 ml/dish of fresh DMEM and 48-h CMs were collected.

2.4. ALP assay

ALP activity in the cultured CPAE cells was determined by methods described previously [18]. Briefly, in the 'DOC extraction method', cultured cells in the collagen-coated dishes were extracted with cold aqueous 1% Na-deoxycholate (DOC) solution, then aliquots of the extracts were incubated with an aqueous *p*-nitrophenylphosphate solution. Incubation was stopped with 0.01 N NaOH. ALP activity were determined by measurement of absorbance at 405 nm and referred to as international units (IU) [18]. In the 'direct in situ assay', CPAE cells were seeded in a 96-well collagen-coated plate at a cell density of 4×10^4 cells/well in 100 μl of the EMEM together with 100 μl of the sample and cultured for 4 days. Cells were drained for the media and washed with 0.9% saline, then incubated with 25 μl/well of the *p*-nitrophenylphosphate solution for 30 min. Incubation was stopped with 0.01 N NaOH, and absorbance at 405 nm was measured with a 96-well multiphotometer. The ALP-inducing activity in the 100 μl sample that gave 1 OD at 405 nm in the direct in situ assay was defined as 1 alkaline phosphatase induction unit (AIU). The activity was corrected for the basal value obtained with the control medium culture.

2.5. Hollow-fiber culture and preparation of serum-free CM

Hs683 cells (3×10^8 cells) were inoculated into a hollow fiber culture system (Cell-Pharm bioreactor system, Unisyn Fibertec Corp., CA, USA) using three BR130 bioreactors with a 10 kDa molecular weight cut-off. Bioreactors were maintained at 37°C and perfused with 2 liters of WRC935 medium (Grace & Co., MA, USA) with 10% FCS. Every day, the media in the extra-capillary space (about 20 ml in total) and in the reservoir were replaced with new ones. As the continual serum-free culture caused marked decrease in the yield of the ALP-inducing activity, the media in the extra-capillary space were replaced with serum-free or serum-containing WRC935 media alternately every day. Serum-free CMs (150 ml) obtained as a 100-fold concentrated solution from 18- to 50-day culture were frozen at −20°C until use.

2.6. Purification

CM (136 ml) from hollow fiber culture was dialyzed against cold 3.3 mM phosphate buffer pH 7.75 with 1 mM EDTA, aprotinin (2 mg/l), chymostatin (2 mg/ml), and leupeptin (0.5 mg/l). The material was applied to an anion-exchange filter (DEAE-Memsep 1010, Millipore Corp., MA, USA) equilibrated with the above dialysis buffer at 4°C. The fractions that passed through were concentrated 20-fold by spin dialysis with Centriprep concentrators (Grace & Co.) and equilibrated in 20 mM phosphate buffer pH 6.0 by gel filtration (PD10 column, Pharmacia LKB Biotechnology, Uppsala). The material was applied to a cation-exchange HPLC column (TSK-Gel SP-5PW 7.5 × 7.5 mm; Tosoh, Tokyo) equilibrated with 20 mM phosphate buffer, pH 6.0, and eluted with a linear gradient of 0 to 0.25 M NaCl. Fractions of 1 ml were collected and 2 μl aliquots were assayed for ALP-inducing activity, and the active fractions were applied to a reversed-phase HPLC column (Cosmosil C4, 4.6 × 250 mm; Nacalai Tesque, Kyoto), and eluted with 0.1% trifluoroacetic acid with a linear gradient of 20–50% acetonitrile. Fractions of 0.5 ml were evaporated to dryness by N_2 gas flashing, and redissolved in TPBS (0.15 M phosphate buffered saline plus 0.005% Tween 20). One-μl aliquots were assayed and active fractions were applied to a HPLC-gel filtration column (TSK-Gel G3000SW, 7.5 × 600 mm; Tosoh, Tokyo) and eluted with TPBS and fractionated in 0.25 ml. Active fractions were collected and subjected to protein analyses.

2.7. Affinity chromatography

Anti-IL-6 antibody column was prepared from *N*-hydroxysuccinimide-activated Sepharose (HiTrap, Pharmacia) according to the manufacturer's manual using anti-IL-6 monoclonal antibodies (Genzyme). CM from the hollow-fiber culture (25 ml in 10% FCS) was applied, and adsorbed material was eluted with 0.1 M glycine, HCl buffer, pH 2.6. The eluted material (about 50 μg) was neutralized, and a portion was applied to the HPLC-gel filtration column.

2.8. Protein analyses

Protein concentrations were measured with a protein assay reagent (BCA kit, Pierce, IL, USA). The purified protein was analyzed by SDS-PAGE in 20% polyacrylamide gel using a PhastSystem (Pharmacia). Amino acid analysis of the purified protein was done with an automatic analyzer (Hitachi Model L8500, Hitachi) after dried purified protein (35 pmol) was mixed with 50 μl of 4 M methansulfonic acid, and hydrolyzed at 110°C for 24 h under reduced pressure. The amino terminal sequence of the purified protein (200 pmol) was determined with a liquid-phase automatic amino acid sequencer (Applied Biosystems Model 477A, CA, USA).

2.9. Enzyme-linked immunosorbent assay (ELISA)

IL-6 concentration was determined by a sandwich enzyme immunoassay in 96-well microtiter plates (Predicta Interleukin-6 kit, Genzyme Corp.).

3. Results

The specific activity of ALP in CPAE cells cultured with the Hs683 CM increased with the number of days of culture (Fig. 1). The ALP activity began to increase markedly after confluency of the cells at 4–6 days in culture. The ALP level in CPAE cells cultured without CM was lowest at day 4 to 5, reflecting their basal unstimulated levels. The specific activity of ALP rose in 14-day-cultured CPAE cells to about 1,000-times higher than these basal levels, and was comparable to the level in isolated brain capillary endothelial cells [18]. The ALP activity was elevated even after 1 day of culture of the CPAE cells with the CM, and the addition of non-cytotoxic levels of actinomycin D (30 ng/ml) and cycloheximide (4 μM) to the culture suppressed 84% and 100% of this elevation, respectively, suggesting the involvement of ALP protein synthesis in the increase in the ALP activity. The factor that induced the ALP activity did not bind to heparin and was heat-labile; this factor was stable on treatment at 56°C for 30 min, while its activity was reduced to 15% of the original on treatment at 70°C for 30 min. The apparent molecular weight on gel filtration of the ALP-inducing factor in the serum-free CM ranged between 20 kDa and 40 kDa with a peak of about 21 kDa.

The ALP-inducing factor was purified from serum-free culture in a hollow fiber culture process by four steps

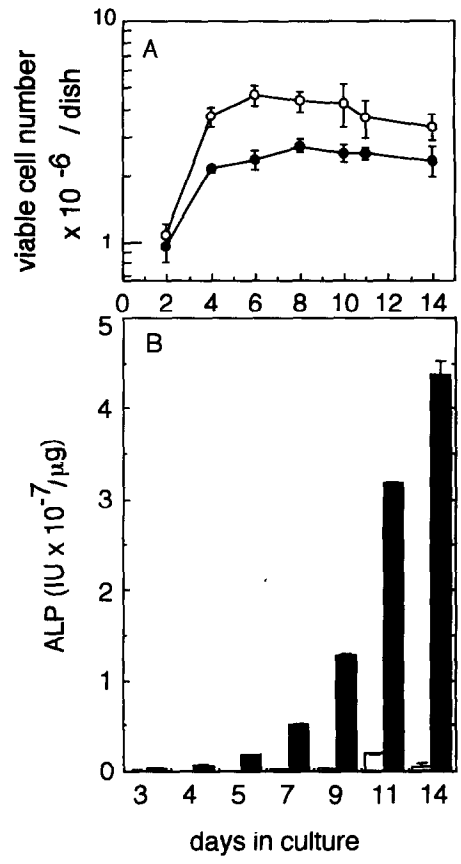

Fig. 1. ALP activity in cultured CPAE cells induced by CM of Hs683 glioma cells. (A) Growth of CPAE cells cultured with (●) or without (○) 50% CM of Hs683 cells. Abscissa: days in culture. (B) Specific activity of ALP in CPAE cells cultured with (■) or without (□) 50% CM of Hs683 cells. ALP activity in the DOC extract was determined as described in section 2. Mean ± S.E.M. of 4 assays.

of chromatography (Table 1). Final gel filtration on HPLC showed a single peak with a molecular weight of 21 kDa with ALP-inducing activity. The SDS-PAGE analysis of the purified protein showed a single band with an apparent molecular weight of 24 kDa (Fig. 2). The amino acid sequence of the amino terminus of the purified protein was VPPGEDSKDVAAPXXQPL (one letter presentation of amino acids, X is unidentified). Apart from this main sequence, a minor sequence, GEDSKDVAAP was also detected; the yield was 14% of the total purified protein. Homology search of these amino terminal sequences showed that they were both identical to the partial sequence of human IL-6 deduced from the cDNA sequence [19]. The amino acid composition of the purified protein substantially agreed with that of human IL-6 (Table 2) [19]. This purified protein reacted with two commercially available monoclonal antibodies to human IL-6. The IL-6 content estimated by ELISA using a monoclonal anti-IL-6 antibody and the ALP-inducing activity were almost parallel at each step of purification (Table 1). Also, IL-6 in the CM was purified more easily by two-step procedures: affinity chromatography and gel filtration (for details, see section 2).

Thus, an induction factor in the CM was identified as IL-6 by biochemical analyses of the purified protein, i.e., molecular weight (24 kDa by SDS-PAGE, and 21 kDa by gel filtration) [20], amino-terminal sequence, amino acid composition, and immunoreactivity to anti-IL-6 antibodies of the purified protein. Moreover, a recombinant preparation of IL-6 (rIL-6) had similar ALP-inducing activity in CPAE cells; it was potent at ng/ml concentrations (Fig. 3). IL-6 content and the ALP-inducing activity of CMs of various human glial cell lines and other non-glial cell lines (see section 2.2) were determined. All glial cell lines examined had the ALP-inducing activity (data not shown). The CM of Hs683 glioma cells had the highest content of IL-6 (75 ng/ml) together with the highest ALP-inducing activity (192 AIU/ml) among the cell lines examined.

4. Discussion

Astroglia and microglia produce a large amount of IL-6 on brain injury and inflammation [20–22]. IL-6 is presumed to aid survival and differentiation of neurons and regeneration of vessels [20]. Capillary endothelial cells newly formed in the injured area first show no or weak ALP activity and higher permeability [7]. After two weeks, these immature endothelial cells grow into the mature type with concomitant appearance of high ALP

Table 1
Purification of ALP-inducing activity in Hs683 CM from hollow-fiber culture

Purification step	Protein (mg)	Activity (AIU)	Specific activity (AIU/mg)	Purification (-fold)	Yield* (%)	IL-6** (μg)	Yield*** (%)
Medium[a]	89.92	847552	9426	1		490	
DEAE	44.85	858000	19130	2	101	366	75
SP	1.20	196333	163611	17	23	82	17
C4	0.10	20865	208650	22	2	ND	ND
G3000SW	0.02	15142	757100	80	2	11	2

[a]Serum-free CM (136 ml) from hollow-fiber culture. *Yield of ALP-inducing activity. **IL-6 content estimated by ELISA. ***Yield of immunoreactive IL-6. ND, not determined.

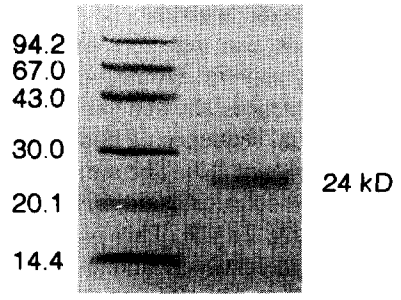

Fig. 2. SDS-PAGE analysis of the protein purified by four-step chromatography. The purified protein, which was electrophoresed under a reduced condition in 20% polyacrylamide gel, showed single band with an apparent molecular weight of 24 kDa (right lane). The chromatography is summarized in Table 1.

activity [7]. IL-6 secreted by activated astrocytes may induce ALP activity in the endothelial cells under certain pathological conditions such as infarct, injury, and inflammation.

Glucocorticoid, an anti-inflammatory mediator, induces ALP activity in cultured murine endothelial cells, and IL-1 suppresses this induction [23]. Taken together with our findings, local concentrations of IL-1, IL-6 and glucocorticoid may modulate the level of ALP activity detected in endothelial cells in peripheral tissues [24] as well as in the brain.

Primary-cultured astrocytes and cultured cell lines of glioma and glioblastoma have been shown to secrete IL-6 [20,25,26]. CM from primary-cultured astrocytes is known to induce development of tight junctions of BBB type in cultured CPAE cells [10,14]. CMs of C6 rat glioma and human astrocytoma cells caused suppression of the permeability of cultured brain microvascular endothelial cells [13,15]. IL-6 seems unlikely to be responsible for these inductions, because it rather increases permeability in cultured carotid endothelial cells [27]. Other molecules, e.g. catecholamine [13], or glia-derived unidentified factors [9,12,16] might have a stronger influence on the endothelial permeability than IL-6.

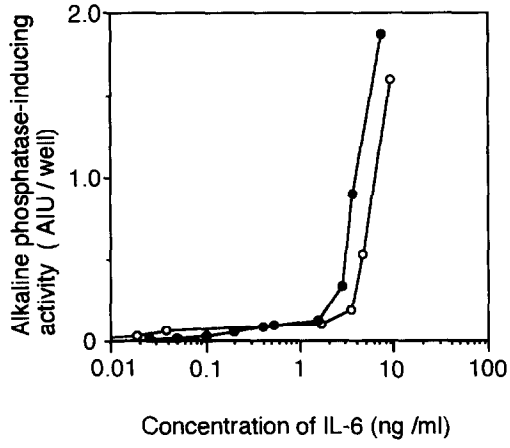

Fig. 3. ALP-inducing activity of the purified protein (○) and rIL-6 (●) for CPAE cells. CPAE cells cultured in a 96-well plate for 5 days with the purified protein or rIL-6 were assayed for ALP activity by direct in situ assay. The abscissa indicates the final concentrations of IL-6 in the culture, which were determined by ELISA. The purified protein and rIL-6 showed similar potency at ng/ml concentrations.

Minute amounts of IL-6 message are expressed constitutively in the brain even under normal conditions [28,29]. The levels of IL-6 message in the brain are highest at postnatal age 20 days [29]. ALP activity in the brain, which is mostly derived from microvascular endothelial cells, is also highest on similar postnatal days [6,30]. This means that concomitant peaks of IL-6 message and ALP activity occur in the brain during ontogenesis. Our present findings, together with these observations, raise the possibility that minute amounts of IL-6 secreted by astrocytes induce and maintain ALP activity in the microvessels and are responsible for late phase maturation or terminal differentiation of the BBB endothelial cells [2,6].

IL-6 signals the terminal differentiation of B cells [20], resulting in high ALP activity in plasma cells [31]. Our present findings on the endothelial cells suggest a closer relationship between IL-6 signaling and ALP induction. This view is now supported by sequence analysis on the liver/bone/kidney-type ALP gene [32], which both plasma cells and endothelial cells express [31,33]. The NF-IL6-binding sequence (TGGAGACAAA), an IL-6 responsive element, which is a motif sequence found on many genes regulated by IL-6 [34], was present upstream (−592 to −583) of the ALP gene.

Table 2
Amino acid composition of the purified protein

Amino acid	nmol	Composition (Lys = 14)	Theoretical value (IL-6)
Ala	0.463	13.4	13
Arg	0.279	8.1	9
Asx	0.666	19.3	18
Glx	0.843	24.4	30
Gly	0.231	6.7	5
His	0.071	2.1	2
Ile	0.26	7.5	9
Leu	0.693	20.1	23
Lys	0.484	14	14
Met	0.188	5.4	5
Phe	0.224	6.5	7
Pro	0.158	4.6	7
Ser	0.612	17.7	15
Thr	0.421	12.2	12
Trp	ND	ND	1
Tyr	0.05	1.5	3
Val	0.099	2.9	6
1/2Cys	0.11	3.2	4
Total	5.852	169 18902*	183 20835*

*Molecular weight. ND, not detected.

Our present findings provide a basis for understanding endothelial cell–astrocyte interaction and the involvement of cytokines in it.

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