Expression of factor X in both the rat brain and cells of the central nervous system

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Received 22 September 1999; received in revised form 18 November 1999

Edited by Shozo Yamamoto

Abstract Prothrombin is expressed in the central and peripheral nervous systems. However, the mechanism responsible for the activation of prothrombin to thrombin by the activated form of factor X in the central and peripheral nervous systems remains to be explored. Here, we investigated the expression of factor X mRNA in the brain and some cell lines derived from the central nervous system. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated the expression of mRNA encoding factor X in the rat brain, A172 (human glioblastoma) and GOTO (human neuroblastoma) cells. The sequences of PCR-derived fragments were identical to those reported for rat and human factor X. These results indicated the synthesis of factor X in the cells of the central nervous system.

Key words: Factor X; Brain; Central nervous system; Thrombin; Prothrombin

1. Introduction

Thrombin regulates a number of important biological responses of cells of the central nervous system (CNS) [1] and the peripheral nervous system [2]. These cellular reactions occur at picomolar concentrations of thrombin and are mediated via thrombin receptors and subsequent signaling [3]. Prothrombin is endogenously expressed in the CNS [4], but the mechanism responsible for prothrombin activation by factor Xa and the source of the precursor of factor Xa, prothrombin activator, in the central and peripheral nervous systems has not been investigated.

Factor X is synthesized in the liver [5] and in the peritoneal macrophages [6]. Factors X/Xa have also been identified in tumor cells and macrophages by immunohistochemical studies [7]. Northern blotting analysis demonstrated that factor X mRNA was present in various tissues but not in the brain [8]. On the other hand, anti-factor X/Xa antibodies stained factor X/Xa in epithelial cells, macrophages in the white matter of the brain and brainstem neurons [9]. However, production of factor X in cells in the CNS remains to be determined.

Here, we examined the presence of factor X mRNA in the rat brain and several cell lines derived from CNS cells by RT-PCR and demonstrated the expression of factor X in both the brain and cells of the CNS.

2. Materials and methods

2.1. Cell culture

A172 human glioblastoma, GOTO human neuroblastoma, T98G human glioblastoma, HepG2 human hepatocellular carcinoma and U373 human astrocytoma cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (Cell Culture Technologies) and 70 μg/ml kanamycin. Cultures were kept in a 5% CO_2/95% humidified air incubator at 37°C. Cells were plated on tissue culture dishes (Iwaki Glass, Japan) and passaged after trypsinization every 5–6 days.

2.2. Animals

Adult Wistar rats were anesthetized by intraperitoneal injection with barbiturate and perfused with phosphate-buffered saline to remove the monocytes and macrophages in the blood. Then, the cerebrum, cerebellum and liver were dissected out. Tissues were stored in liquid nitrogen until preparation of RNA.

2.3. RNA preparation

Total RNA was prepared by the standard acid guanidinium isothiocyanate-phenol-chloroform extraction procedure [10] using a kit (Isogen, Nippon-Gene, Japan). RNA thus obtained was electrophoresed on 1% agarose gels in the presence of 6% formaldehyde to assess its purity.

2.4. cDNA preparation and PCR analysis

Oligonucleotide probes were designed based on the published sequences of human factor X cDNA [11] and rat factor X cDNA [12] divided into four and five regions for optimal amplification, respectively. For amplification of human and rat factor X, primers were obtained commercially (Amersham Pharmacia Biotech). Primers for the G3PDH gene were utilized as controls (Clontech). Total RNA (2 μg) was subjected to cDNA synthesis using oligo $(dT)_{12-18}$ primer and AMV reverse transcriptase (Invitrogen) after treatment with DNase (Nippon-Gene, Japan) and re-extraction of RNA. The cDNA samples were diluted in a final volume of 50 μl. PCR was carried out in 25-μl reaction mixtures including 1×PCR buffer, 80 μM dNTPs, 0.25 μM of each 5′ and 3′ primer, 0.5 units of Z-*Taq* polymerase (Takara Shuzo, Japan) and 0.5 μl of synthesized cDNA using a GeneAmp PCR system 9700 (Perkin Elmer) for 40 cycles of 98°C for 3 s, 60°C for 3 s and 72°C for 5 s. PCR products were visualized by electrophoresis (2% agarose) and stained with ethidium bromide.

2.5. Sequence of factor X

Amplified DNA fragments were purified from agarose gels using the quick prep DNA purification system (Bio-Rad). Dideoxy sequencing of DNA fragments was performed using a DNA Sequencing kit (Perkin Elmer) and a nucleotide sequencer (model 373A, Applied Biosystem) to confirm their sequences.

2.6. Quantitative reverse transcription PCR

Competitor DNAs flanked by the target gene primer sequences were constructed using a competitive DNA construction kit (Takara Shuzo). For amplification of human factor X transcripts and their competitors, primers 5′-ACGAGAAGGGCCGGCAGTGTACGGTCATCATCTGACAC-3′ and 5′-GGAGAGGACGTTATGACCTCCGAATACATCAAACGCGCGGAC-3′ for human factor X (nucleotides 1103–1457, 355 bp corresponding to serine protease

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PII: S0014-5793(99)01657-9

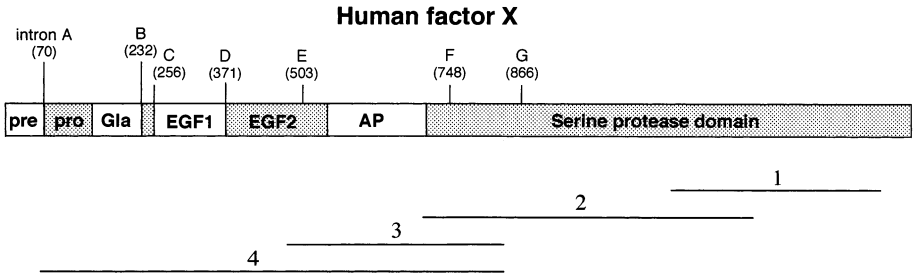

Fig. 1. RT-PCR of human factor X. The starting position of each primer in relation to the mRNA structure is shown above the figure. Primers were designed to generate PCR products of under 1 kb. Primers 2, 3 and 4 were designed to span an intron in the factor X gene.

region), 5′-CTCACTGCCGCCCATTGTCTCGTACGGTCATCAT-CTGACAC-3′ and 5′-GGCCGTGACCTTCGTGTAGATGCGCG-TGAGTATTACGAAGGTG-3′ for rat factor X (nucleotides 823–1117, 295 bp corresponding to serine protease region) were used. Aliquots of 5 μl of competitor DNAs were coamplified with 0.5–2 μl of cDNA under the same conditions as described above. The PCR products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide. The band intensities were quantified using an Electrophoresis Documentation and Analysis system 120 (Kodak) and plotted on logarithmic scales, the *x*-axis representing the competitor and the *y*-axis the ratio of target to competitor (Fig. 3).

3. Results and discussion

RT-PCR analysis was performed to confirm the expression of factor X in A172 (human glioblastoma), GOTO (human neuroblastoma), T98G (human glioblastoma) and U373 (human astrocytoma) cells. As a positive control, analysis was performed with HepG2 (human hepatocellular carcinoma) cells, which are well known to produce factor X. The expression of factor X mRNA encoding from the pre-pro to serine protease region corresponding to the mature protein (Fig. 1) was detected in A172 and GOTO (data not shown). All PCR products were of the expected size and were the same as those amplified in HepG2. We also analyzed the expression of factor X mRNA in the rat cerebrum, cerebellum and liver by a similar method. Signals of factor X were detected from the rat brain (cerebrum and cerebellum) and liver, and the PCR products were of the expected sizes (data not shown). Negative control reactions without reverse transcriptase in the cDNA synthesis process showed no bands in all experiments, indicating that there was no contamination by genomic DNA in the RNA samples.

Next, the sequences of the PCR fragments were analyzed to confirm whether the PCR products were amplified from factor X mRNA. DNA sequences obtained from A172 and HepG2 cells were compared over 69% of the ORF of factor X mRNA and no differences were found. There were no differences in the amplified PCR products from rat cerebrum and liver, at least in the region compared (85.8% of ORF). These results indicated that A172 human glioblastoma cells and rat brain express mature factor X mRNA.

To assess the levels of factor X mRNA in the cell lines and brain, competitive PCR was performed (Fig. 2). GOTO and A172 cells expressed factor X mRNA at approximately 1×10^2 and 4×10^1 copies/reaction (corresponding to 5×10^3 and 2×10^3 copies/μg RNA), respectively (Fig. 3). The expression level of factor X mRNA was at least 100-fold higher (1.3×10^4 copies/reaction, 6.5×10^5 copies/μg RNA) in HepG2 cells. The results of competitive PCR of the control G3PDH gene quantitatively demonstrated that levels of amplified products were equivalent in all cell lines, confirming that the amounts of cDNA subjected to PCR were the same in all cell lines.

We also investigated expression of factor X mRNA in the rat brain with another set of primers (see 'Quantitative Reverse Transcription PCR') and PCR competitor. Factor X was expressed in both the cerebrum and cerebellum at 1×10^2 copies/reaction (5×10^3 copies/μg)(Fig. 4). The level

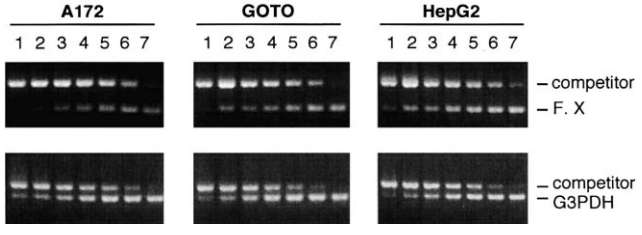

Fig. 2. Comparative evaluation of human factor X mRNA for A172 and GOTO cells by competitive PCR. The mRNA levels of human factor X (upper) and a housekeeping gene, G3PDH (lower), were compared between cell lines. (upper) Lanes 1–7 indicate three-fold serial dilutions of 1×10^4 copies/reaction of factor X competitor DNA for A172 and GOTO cells, 1×10^5 copies/reaction of factor X competitor for HepG2 cells. (lower) Lanes 1– 7 indicate three-fold dilutions of 1×10^6 copies/reaction of G3PDH control gene for all cell lines. Competitors were coamplified with constant amounts of cDNA as described in Section 2.6.

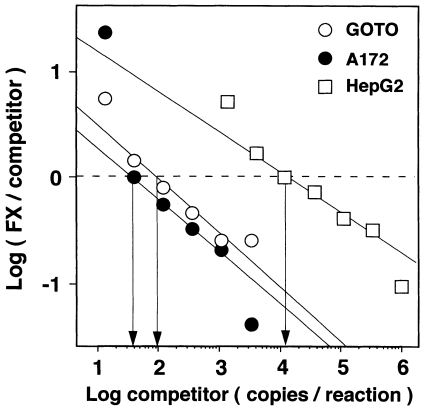

Fig. 3. Data analysis of competitive RT-PCR. The intensity of ethidium bromide-stained DNA bands was quantified and plotted on the *x*-axis, competitor, versus the *y*-axis, log ratio factor X over competitor, on a logarithmic scale.

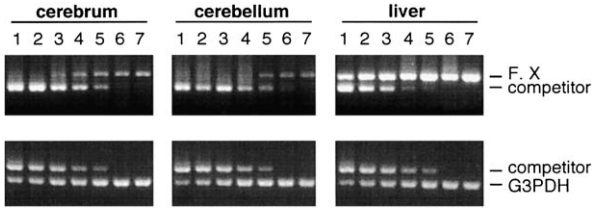

Fig. 4. Comparative evaluation of rat factor X mRNA for the cerebrum and cerebellum by competitive PCR. Experiments similar to those described in Fig. 2 were performed. The mRNA levels of rat factor X (upper) and a housekeeping gene, G3PDH (lower), were compared between cell lines. (upper) Lanes 1–7 indicate five-fold serial dilutions of 1×10^4 copies/reaction of rat factor X competitor DNA for cerebrum and cerebellum, 1×10^6 copies/reaction of factor X competitor for liver. (lower) Lanes 1–7 indicate three-fold dilutions of 3×10^5 copies/reaction of G3PDH control gene for tissues. Competitors were coamplified with constant amounts of cDNA as described in Section 2.6.

of factor X mRNA expression in the liver, the primary site of factor X synthesis, was approximately 1×10^5 copies/reaction (5×10^5 copies/μg RNA); this level was 1000-fold higher than those in the cerebrum and cerebellum.

Based on these observations, we examined the expression of factor X at the mRNA level in the rat brain and human cell lines by RT-PCR analysis, and confirmed the sequence of the PCR products and competitive PCR. Until the present study, the expression of factor X in the brain had not been demonstrated conclusively because of difficulty in avoiding serum contamination. The expression of factor X was not demonstrated in the brain by a Northern blotting method [8]. However, we demonstrated the expression of factor X in the brain and human CNS cell lines using sensitive mRNA detection methods. This is the first evidence that factor X mRNA is expressed in the brain tissue and cultured human cell lines derived from the CNS. We were not able to identify which cell type(s) expressed factor X in this study. However, our preliminary experiments showed that primary cultured astrocytes expressed factor X mRNA (unpublished observation). This implies that the source of factor X in the brain is at least local synthesis by astrocytes.

Factor X expressed endogenously in the CNS should be a component of a prothrombin activator. Recently, we showed that prothrombin is activated to thrombin subsequent to activation of factor X in the cells of extravascular regions such as glial and neural cells by prothrombinase complex [13]. Our observations suggested that activation of prothrombin on intact cells such as glial and neural cells occurs in response to nanomolar-order concentrations of factor Xa [13]. Picomolar-order concentrations of thrombin also cause significant physiological responses [3]. The optimal concentration of thrombin to cause the biological reaction in CNS cells via the thrombin receptor is far lower than that for fibrin conversion in plasma. This could induce the activation of prothrombin by factor Xa, produced in the CNS, even if the level of factor X transcript expression in the brain (cerebrum and cerebellum) is 100–1000-fold lower than that in hepatic cells. The optimal concentration of thrombin to cause the biological reaction in CNS cells via the thrombin receptor appears to be entirely different from that for fibrin conversion in plasma.

Prothrombin is physiologically activated to thrombin by a prothrombinase complex consisting of factor Xa, factor Va, Ca^{2+} and a phospholipid-containing surface. Together the constituents of prothrombinase enhance the factor Xa-mediated activation of prothrombin to thrombin by five orders of magnitude. Factor V(a) may also participate in the composition of prothrombinase complex in the CNS, although it has not been confirmed that factor V is present in the CNS. Human brain pericytes expressed effector cell protease receptor-1 (EPR-1) and propagated blood coagulation including the extrinsic pathway [14]. Thus, EPR-1 might contribute to the molecular assembly of the components of prothrombinase on CNS cells.

Alternative cleavage of factor X by plasmin and its final products was also reported to inhibit blood coagulation [15], suggesting that plasmin is a regulator of the function of factor X. Factor X in the brain may participate in fibrinolysis or have other as yet unknown functions when plasmin is expressed in the brain.

Acknowledgements: We are grateful to Dr. Yongchol Shin for his helpful suggestions. We would also like to thank Yusuke Takahashi, Takayoshi Ashie, Yukako Shimano and Satsuki Hori for technical assistance. This work was supported by a Research Grant for Cardiovascular Diseases (8A-1) from the Ministry of Health and Welfare to T. Morita.

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