Cloning of aminopeptidase N promoter and its activity in hematopoietic cell and different tumor cell lines

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Abstract **Aminopeptidase N (APN) promoter region was cloned and sequenced from peripheral blood mononuclear cells. The recombinant reporter construct containing the promoter and luciferase gene, designated pXP1-APNLuc, was introduced into myeloblastic cell line, T lymphocyte cell line and various tumor cell lines. Luciferase assay showed that APN upstream promoter is myeloid-specific for high expression in myeloblastic cell line and much lower expression in T lymphocyte cell line. The promoter activity was relatively high in lung adenoma cell line compared with other tumor cell lines including hepatoma cell line, tong cancer cell line and esophageal cancer cell line in which the promoter activity significantly diminished or was almost undetectable. The characteristics of APN promoter may provide a new strategy for specific myeloprotection while tumor patients are being treated with chemotherapy and/or radiotherapy.**

Keywords: aminopeptidase N, myeloid-specific promoter, reporter gene.

Chemotherapy and radiotherapy are two of the mainstays of current synthetic therapy for tumor patients. However, myelosuppression induced by anticancer agents and irradiation is one of the major side effects that limit the effectiveness of the treatments. Introduction of drug resistance genes and irradiation resistance genes into hematopoietic precursors leading to protection against the hematotoxicity has been proposed to reduce myelosuppression[1,2]. The administration of the genes is capable of protecting not only hematopoietic cells but also the tumor cells invaded into bone marrow since the expression of the exogenous genes is not specific. One of the solutions to this problem is to develop vectors containing specific promoter elements that regulate the expression of drug- or radiation-resistance genes only in hematopoietic cells.

The expression of aminopeptidase N (APN) is highly lineage-specific in the earliest myeloid progenitor cells and the derived differentiated cells[3]. In the study we cloned the promoter of APN gene and assayed the activity in myeloblastic cell line and different kinds of tumor cell lines.

1 Materials and methods

(ⅰ) Cell cultures. Myeloblatic cell line KG1a (gift from Prof. Zheng Dexian, Institute of Basic Medical Sciences, Peking Union Medical College and Chinese Academy of Medical Sciences) was cultured in Isocove's modified Dulbecco's medium (Gibco). T lymphocyte cell line Jurkat (also kindly provided by Prof. Zheng Dexian), lung adenoma cell line Anip973, and hepatoma cell line BEL 7402 were grown in RPMI 1640 medium (Hyclone). Tong cancer cell line TCA and hepatonoma cell line HepG2 were maintained in Dulbecco's modified Eagle medium / F12 medium (Sigma). Esophageal cancer cell line NEC (gift from Prof. Lu Shixin, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences) was cultured in M199 medium (Gibco). Each medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin plus 100 U/mL streptomycin. The cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2.

(ⅱ) Amplification of APN promoter. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. One μg of DNA purified from the cells was used for PCR. After hot start at 95℃ for 5 min, thirty cycles of PCR were performed: denaturation at 95℃ for 1 min, annealing and extension at 68℃ for 3.5 min, then the extension completed at 68℃ for 7 min. The primers were 5′GATGGATCCTTCTGAACTAGGAGT3′ and 5′TATGAATTCGCTGGAACCTGGACCCT3′ in which a *Bam*HⅠ site and an *Eco*RⅠ site (underlined) were added. The reaction system contained 5% DMSO.

(ⅲ) Plasmids construction. The APN promoter fragment amplified by PCR was cloned into the *Bam*HⅠ and *Eco*RⅠ sites in the pBlueScript Ⅱ SK (Stratagene) to generate a construct named pBSAPN. The plamid was then digested with *Bam*HⅠ and *Hin*dⅢ, the released insert was ligated into pXP1 (gift from Prof. Paul Dobner, University of Massachusetts) to generate the pXP1- APNLuc construct. The cloned fragment was sequenced by the dideoxynucleotide chain termination method. Sequencing primers were T7 and T3 that were complementary to sites flanking the multiple cloning sites in the vector pBlueScript Ⅱ SK.

(ⅳ) Reporter gene analysis. Transfection was performed with 6 μg of the reporter plasmid pXP1-APNLuc and 2 μg of β-gal control plasmid PCH110 (Pharmacia). KG1a and Jurkat cell lines were electroporated with Gene Pulser (BIO-RAD) set at 960 μF and 200 V. BEL7402, HepG2, TCA and NEC cell lines were transfected by DEMRIE-C Reagent (Gibco). Assays to detect luciferase and β-gal activity were performed as described[4]. Preparation of cell extracts and analysis of luciferae and β-gal

activities were carried out using luciferase assay system and β-galactosidase enzyme assay system (Promega) according to the recommended procedures of the manufacturer. Relative luciferase activity = luciferase activity/β-gal activity.

2 Results

(ⅰ) Amplification of APN promoter. It was very difficult to amplify APN promoter because the region was GC rich. In order to destroy the secondary structure of DNA and lower the annealing temperature, DMSO was added to the reaction system and a two-step procedure of PCR was carried out. A single band of 520 bp was produced (fig. 1).

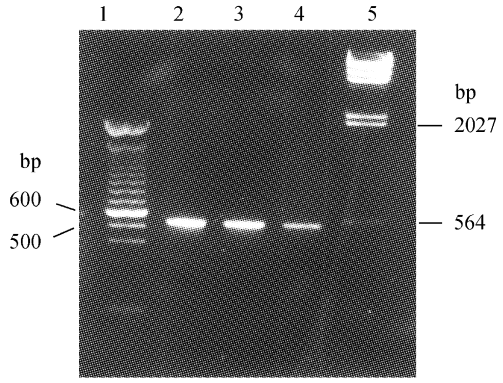

Fig. 1. Amplification of APN promoter. 1, 100 bp DNA marker; 2, 5%DMSO; 3, 2.5%DMSO; 4, 1%DMSO; 5, λ/*Hin*d Ⅲ DNA marker.

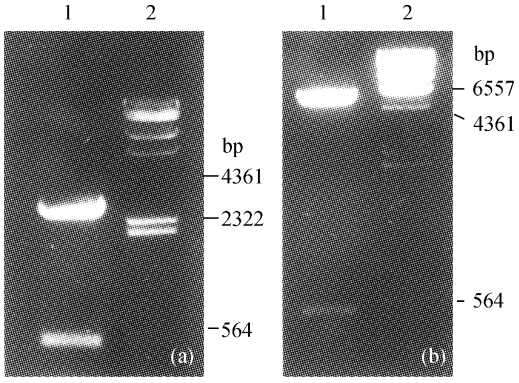

Fig. 2. Vectors construction and identification. (a) pBSAPN identification. 1, *Bam*HⅠ/*Eco*RⅠ digests pBSAPN; 2, λ/*Hin*d Ⅲ DNA marker. (b) pXP1-APNLuc indentification. 1, *Bam*HⅠ/*Hin*d Ⅲ digests pXP1-APNLuc; 2, λ/*Hin*d Ⅲ DNA marker.

(ⅱ) Vector construction and identification. The resulting construct pBSAPN was cut with *Bam*HⅠ and *Eco*RⅠ and a 0.52 kb fragment and a 2.9 kb fragment were produced. After the digestion with *Bam*HⅠ and *Hin*d Ⅲ, a 0.52 kb insert was also released from pXP1-APNLuc. The orientation of the promoter was confirmed by sequencing pBSAPN (fig. 2). These data demonstrated that the size and the orientation of the promoter fragment were correctly cloned in the vector.

(ⅲ) DNA sequencing. The promoter fragment was sequenced with T3 and T7 primers and the sequences from five clones were identical, indicating that the substitutions (see below) in the promoter sequence were not created by the PCR procedure. Compared with the results of Shapiro[5], two differences were detected: a A→G substitution at –192 and a G→A substitution at –82. The differences may represent the polymorphism in APN promoter.

(ⅳ) Reporter gene assay. The reporter gene was expressed at highest level in myeloblatic cell line KG1a and significantly decreased in T lymphocyte cell line Jurkat and other tumor cell lines. The relative luciferase activity in BEL7402, HepG2, Jurkat, NEC, TCA, 44.5% and Anip973 accounted for 3.9%, 3.5%, 15.9%, 40.5% and 83.1% of the activity in KG1a, respectively (fig. 3).

3 Discussion

APN is a member of the family of membrane-bound metallopeptidases. APN gene is encoded by 20 exons that extend more than 30 kb on long arm of human chromosome 15[6]. Two physically distinct promoters, 8 kb apart from each other, are located on APN genomic DNA. APN driven by upstream promoter can be detected on the earliest progenitor cells committed to the granulocyte/monocyte developmental pathway as well as its more differentiated progeny, but is not found on normal hematopoietic of other lineages or their progenitors while downstream promoter acts in liver, small intestine and endometrium[7—9]. The sequence within upstream promoter contains elements that regulate myeloid-specific gene expression.

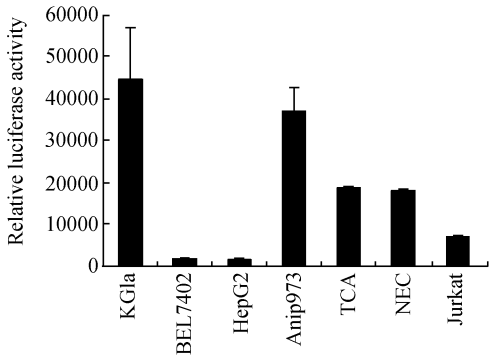

Fig. 3. Activity of APN promoter in myeloblastic cell line KG1a and various tumor cell lines.

The genomic fragment extending from –411 to –291 contains a Myb binding site and three Ets binding sites that would result in a significant reduction of the promoter activity if mutated or deleted. In this study, APN myeloid promoter is cloned by PCR and the sequence shows that the fragment of – 411— –200 which is highly associated with myeloid-specific expression is conservative, in agreement with the results of Shapiro although the polymorphism is present in the promoter region[5]. Reporter gene analysis confirms that the cloned promoter sequence is myeloid-specific for the high level of activity in myeloblastic cell line KG1a and much lower level of activity in T lymphocyte cell line Jurkat. The results of transient transfection also revealed that the promoter activity reduced significantly in tong cancer cell line, esophageal carcinoma cell line and especially in hepatoma cell lines. But the promoter activity is relatively high in lung adenoma cell line compared with other tumor cell lines tested. The utilization of upstream promoter in lung may explain this observed phenomenon[10,11].

In the treatments of breast carcinoma, ovarian carcinoma and meloma, introduction of drug-resistance genes and irradiation-resistance genes into myeloid cells is an effective approach for preventing myelosuppression[2,12—14]. However, there are some limits: the nonspecific expression of exogenous genes also results in increased drug and radiation resistance of tumor cells, thus, this nonspecific approach can only be used for the patients whose tumor cells have not invaded into bone marrow. Clinically, most of tumor patients at diagnosis are at the middle or later stage and tumor cells have already invaded into bone marrow, which are the obstacles of wide clinical use. Application of APN myeloid-specific promoter leading to high expression of drug-resistance genes and irradiation-resistance genes in hematopoietic cells and low or not expression in tumor cells would overcome the limits and play an important role in the future gene therapy for the patients with hepatoma and other tumors.

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