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Butyrate-induced phosphatase regulates VEGF and angiogenesis via Sp1

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ABSTRACT

Sp1 is a ubiquitous transcription factor and master regulator of various eukaryotic gene expression. Better understanding of the role of increased Sp1 levels on angiogenic regulation and the regulatory regions of that transcription factor may act as a useful target in 'transcriptional therapy'. At the molecular level, butyrate inhibits Sp1-DNA binding activity by promoting Sp1 protein dephosphorylation in EAT cells. It also inhibits Sp1 binding activity and reduces expression of VEGF gene, thereby inhibiting angiogenesis. It was confirmed that butyrate induces expression of a tyrosine phosphatase by RT-PCR, cDNA sequence analysis, protein ESI-MS analysis and protein sequence homology comparison. Thus our result strongly suggests that inhibition of angiogenesis by butyrate involves Sp1 dephosphorylation and down-regulation of VEGF gene expression. Further, butyrate inhibits neoangiogenesis induced by tumor cells and VEGF in peritoneum of EAT bearing mice and rat cornea.

Introduction

Sp1 transcription factor is a master regulator of eukaryotic gene expression and one of the very first cellular transcription factors to be identified and cloned [1]. Sp1 is a sequence-specific transcription factor that recognizes GC boxes [2] which are common regulatory elements in promoters of housekeeping genes as well as many tissue-specific genes [3]. The growth of solid tumors and the formation of metastasis are dependent on the formation of new blood vessels [4]. Vascular endothelial growth factor (VEGF) has been identified as a key mediator of tumor angiogenesis [5–6]. Sequence analysis of the VEGF gene promoter revealed several binding sites for transcription factors AP-1, AP-2, Sp1 etc. [7], which are candidate mediators of various signals. Sp1, Sp3 and Sp4 are transcription factors that regulate cell proliferation, vascular endothelial growth factor expression and its receptors (VEGFR1, VEGFR2, VEGFR3) and are over expressed in many cancer cell lines [8]. Sp1 activities are regulated either by post-translational modification or alteration of Sp1 protein. The principle known post-translational modifications are phosphorylation, glycosylation and acetylation [9]. VEGF promoter activity is preceded by the activation of transcription factor Sp1 [10]. Therefore it is clear that a constitutive Sp1 activation is essential for the differential over expression of VEGF, which in turn plays an important role in angiogenesis and the progression of cancer.

Butyrate, a short-chain fatty acid produced during the fermentation of dietary fiber by endogenous intestinal microflora is currently being evaluated as an anti-neoplastic therapeutic agent [11] and clinical trials of butyrate and its derivatives in cancer patients have been already initiated [12–13]. The inhibitory role of butyrate against tumor growth is mediated through a direct effect on tumor cells that results in altered gene expression [14–15]. Transcription factors are usually the final target of many signal transduction pathways and plays an important role in butyrate signaling [16–17]. We have previously demonstrated that butyrate down regulates VEGF/KDR gene expression and inhibits angiogenesis [18]. Further we have shown that there was induction of phosphatase in EAT cells upon butyrate treatment [19]. The aspect of involvement of butyrate induced phosphatase in regulation of VEGF gene expression through transcription factors has not been delineated. The present study was undertaken to study the involvement of Sp1 transcription factor and butyrate-induced phosphatase in the inhibition of angiogenesis through regulation of VEGF gene expression.

Materials and methods

Materials

Ehrlich ascites tumor (EAT)[1] cells were routinely maintained in Swiss albino mice in the animal house, University of Mysore, Mysore, India. Human embryonic kidney (HEK-293) cells were purchased from NCCS, Pune, India. Dulbecco's modified Eagle's medium

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[1] *Abbreviations used:* EAT, Ehrlich ascites tumor; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; BLAST, Basic Local Alignment Search Tool; β-Gal, Beta galactisidase.

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(DMEM), fetal bovine serum (FBS), penicillin–streptomycin and trypsin–EDTA were purchased from Invitrogen, USA. *n*-butyrate was procured from SRL, Mumbai, India. Calcium phosphate, mammalian transfection kit and β galactisidase assay kit were obtained from Stratagene, USA. Luciferase assay kit was from BD Biosciences, USA. T4 polynucleotide kinase kit was obtained from Amersham biosciences. RT-PCR kit was procured from Qiagen, USA. The antibodies to Sp1, p-Ser, p-Tyr, p-Thr and Sp1 oligonucleotides (5′-d (ATTCGA TCG GGG CGG GGCGAG C)-3′) for gel shift assays were obtained from Promega. Radioactive γ-[^{32}P] ATP, α-[^{32}P] dATP and [^{3}H] thymidine were obtained from BARC, Mumbai, India. All other chemicals and reagents were of highest grade commercially available.

Methods

Cell culture and growth of EAT cells in vivo

EAT cells and untransformed HEK-293 cells were cultured *in vitro* in DMEM with 10% FBS, and 1% Penicillin–streptomycin. The cells were cultured in 25 cm^3 tissue culture flasks (NUNC, Genetix Biotech Asia, Bangalore, India) and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C. Upon confluency, the cells were trypsinized with 0.025% trypsin/0.01% EDTA and used for the experiments. EAT cells (5X10^6/mouse) were injected intraperitoneally (i.p) into 8- to 10-week-old Swiss albino mice. The mice were given food and water ad libitum. By recording the animal weights, tumor growth was followed.

Butyrate treatment

Butyrate was diluted in normal saline to a final concentration of 10 mM. Cells were treated with different concentrations of butyrate for various time periods. For *in vivo* treatment, butyrate (300 μl of 100 μM/mouse, i.p) was injected into the EAT bearing mice every alternate day after 6 days of tumor transplantation and the mice were sacrificed on the 14th day. The rationale for injecting butyrate on the 6th day after tumor transplantation was to stimulate the natural state where a partially or wholly grown tumor has to be treated.

Cell proliferation assay

The HEK-293 and EAT cells were plated onto 12-well culture plates at 50,000 cells/well and incubated at 37 °C in 5% CO_2 for 48 h in DMEM. After incubation, different concentrations of butyrate was applied on to the wells in duplicates leaving two wells as control, prior to the addition of [^{3}H] thymidine (1 μCi/ml) to all the wells. After 48 h of incubation, the cells were washed with PBS. The HEK-293 cells were trypsinized, and both the cell lines were fixed with 10% ice-cold trichloroacetic acid to precipitate the DNA. The radioactivity was measured in scintillation solution using liquid scintillation spectrometry.

Purification and assay of phosphoprotein phosphatase

Purification of butyrate induced phosphatase was essentially carried out as previously described [19]. The cytosolic fraction obtained was partially purified by chromatography using an anion exchanger. The DEAE-column was eluted by stepwise salt gradient and the fractions with high enzyme activity were pooled, dialyzed, lyophilized and were applied onto a Sephadex G-200 gel filtration column. The activity of phosphoprotein phosphatase was measured by an extremely sensitive non-radioactive method as described previously [20–21]. The total reaction mixture of 2 ml containing 10 mM Tris–HCl (pH 8.0), TPA activated cell membranes as substrate (30 μg) and enzyme (100 μg) was incubated at 37 °C for 15–20 min. The reaction was stopped by placing the tubes on ice and to an aliquot of the reaction mixture (0.5 ml), malachite green reagent was added and then the tubes were allowed to stand for 10 min. The absorbance was read at 620 nm. Suitable controls were also included. The corrected values of optical density (O.D) at 620 nm were obtained after subtracting the additive O.D. of enzyme alone and substrate alone from the O.D. obtained with enzyme and substrate together. A standard curve of absorbance at 620 nm of phosphate (0–25 nmoles) was determined in parallel.

RT-PCR, cDNA sequencing and homology-identity analysis

Total RNA was extracted from EAT cells treated with and without butyrate (10 mM) at regular time intervals from 0 to 4 h using an RNeasy kit according to the instructions from the manufacturer (Qiagen, USA). Consensus primer sequence (Sal I "GGTCGAC TTTTGGIIIATGIIITGGGA" "CATTGTTCIGGIGTIGGATCCG" BamHI) for known tyrosine phosphatase was synthesized and was obtained from Sigma–Aldrich, India. Amplification of cDNA encoding phosphatase was carried out by Qiagen one-step RT-PCR kit. The RT-PCR product was run on 1.5% agarose gel. The DNA fragments from the agarose gel were extracted using Qiagen gel extraction kit.

Sequencing of cDNA was carried out using ABI prism genetic analyzer (NCBS, Bangalore, India). The cDNA sequence homology search was done by Basic Local Alignment Search Tool (BLAST).

Trypsin digestion and mass spectroscopy

Butyrate induced phosphatase was isolated using the procedure previously described [19]. The protein was run on SDS–PAGE and stained with silver nitrate. The gel was washed with 5% acetic acid for 5 min to ensure complete termination of development. The band was excised, digested with trypsin and subjected to mass spectrometric electro spray ionization mass spectrometry (ESI-MS, NCBS, Bangalore). The obtained peak profile was analyzed by Mascot peptide mass mapping tool software.

Immunoprecipitation and western blot analysis

Nuclear proteins (500 μg) from control EAT cells and EAT cells treated with butyrate for 60,120 and 180 min, respectively, were immunoprecipitated with 4 μg of Sp1 antibodies and 30 μl of Protein A-agarose in binding buffer overnight at 4 °C. The Sp1-antibody-Protein A complexes were centrifuged at 4000 rpm and the pellets were washed 5 times with binding buffer. The IP-Sp1 protein was resuspended in western blot sample buffer, boiled, electrophoresed on SDS–PAGE and transferred onto nitrocellulose membrane. The immunoblots were developed with antibodies against phosphoserine, phosphothreonine, phosphotyrosine. Separate nuclear proteins (50 μg) were used for western blotting to detect Sp1 abundance in nucleus (16).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted by the procedure previously described [22] from EAT cells treated with and without butyrate for 60,120 and 180 min, respectively. The double stranded Sp1 consensus oligonucleotide probes [5′-d (ATT CGA TCG GGG CGG GGC GAG C)-3′] were end-labeled with γ-[^{32}P] ATP. Nuclear proteins (40 μg) was incubated with 40fmoles of γ-[^{32}P]-labeled Sp1 consensus oligonucleotides for 30 min in binding buffer containing 100 mM HEPES (pH 7.9),10 mM $MgCl_2$, 125 mM KCl, 0.5 mM EDTA, 4% glycerol,0.5% NP-40,1 μg of poly [dI-dC] and 1 mg/ml BSA. The specificity of the Sp1 DNA binding was determined in competition reactions in which a 40-fold molar excess of unlabelled Sp1 oligonucleotide was added to the binding reaction 20 min prior to the addition of radiolabeled probes.

In the super shift assay, antibody (1 μg) reactive to mouse Sp1 protein was added to the reaction mixture immediately after the addition of radio labeled Sp1 probes. The samples were electrophoresed in 4% non denaturing polyacrylamide gel in 0.5% TBE at room