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TNFα induces tolerant production of CXC chemokines in colorectal cancer HCT116 cells via A20 inhibition of ERK signaling

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ABSTRACT

Ubiquitin editing enzyme A20 functions as a tumor suppressor in various cancer. However, the mechanism for A20 regulation of cancer progress is not fully understood. In this study, we found that in human colorectal cancer HCT116 cells, TNFα induced a tolerant production of CXC chemokines, including CXCL1, 2, and 8 in a dose and time dependent manner. TNFα pre-treatment of HCT116 cells down-regulated the chemokine production induced by TNFα re-treatment. TNFα induced the phosphorylation of MAPKs ERK, JNK, P38 and NF-κB P65, but only ERK inhibition decreased TNFα-induced chemokine production. Both RT-PCR and FACS results showed that TNFα treatment did not regulate the expression of TNF receptors. However, TNFα up-regulated the expression of A20 at both mRNA and protein levels significantly. TNFα pre-treatment inhibited the signal transduction of MAPKs induced by TNFα re-stimulation, and A20 over-expression decreased the signal transduction of ERK and P38. Meanwhile, A20 inhibition by RNA interference reversed chemokine down-regulation induced by TNFα re-stimulation after TNFα pre-treatment. Taken together, these results suggested that in human colorectal cancer cells, A20 may function to inhibit cancer progression via down-regulation of TNFα-induced chemokine production by suppression of ERK signaling.

1. Introduction

Chronic inflammation and cancer are closely associated in various tissues. The link between chronic inflammation and tumorigenesis was first proposed 100 years ago. Direct evidences for chronic inflammation in various tumor samples are firmly confirmed [1]. Colorectal cancer incidence is increased in ulcerative colitis, and anti-inflammatory agents decrease intestinal malignancies [2]. Patients infected with Helicobacter Pylori have been found to develop more gastrointestinal neoplasms [3]. Hepatocellular carcinoma is more frequent in patients infected with hepatitis C virus [4]. Chronic inflammation is characterized by the infiltration of various kinds of immune cells, including antibody-producing plasma cells, lymphocytes, neutrophils and macrophages, which migrate to inflammatory tissues in response to chemokines and function to phagocytose the cellular debris and microorganisms [5]. In tumor tissues in response to inflammatory media, malignant cells also release chemokines, which in turn recruit various kinds of immune cells to sustain the inflammatory cycle [6].

A20 was initially discovered as a TNFα-induced gene in human umbilical vein endothelial cells, so it is also known as TNFα-induced protein 3 (TNFAIP3) [7]. A20 is an important anti-inflammatory molecule which inhibits multiple intracellular signal pathways [8]. In LPS-primed macrophages, A20 has been found to be up-regulated [9], and A20-IRAK1 interactions are determinants of endotoxin tolerance [10]. In enterocytes, A20 also functions to induce tolerance to LPS [11]. A20 is required to protect mice form endotoxic shock by termination of TLR-induced activation of the NF-κB and TLR-induced expression of pro-inflammatory cytokines via directly removing ubiquitin moieties from signal adaptor TRAF6 [12]. A20 has been reported to function as a tumor suppressor in several lymphomas [5]. In pancreatic cancer tissues, A20 expression is reduced, and low A20 expression is associated with pancreatic cancer behavior [13]. A20 has been reported to restrict Wnt signaling in intestinal epithelial cells and to suppress colon carcinogenesis [14]. By inhibition of Twist1 expression, A20 functions to suppress hepatocellular carcinoma proliferation and metastasis [15].

Chemokines are a family of small, highly conserved, secreted proteins which mediate different biological functions, such as chemotaxis, hematopoiesis, angiogenesis, and wound healing by interaction with a group of seven transmembrane, G protein coupled receptors (GPCRs) [16]. The chemokine family can be further divided into four subfamilies

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depending on the location of the first two cysteine (C) residues in their protein sequence: including CXC chemokines, CC chemokines, C chemokines and CX3C chemokine [17]. CXC chemokines can be subdivided into two subclasses, including ELR(+) chemokines such as CXCL1, 2, and 8, which contain the tripeptide glutamate-leucine-arginine motif and mediate angiogenesis typically via interaction with the CXCR2 receptor, and ELR(−) chemokines such as CXCL9, 10, and 11 which absent the tripeptide glutamate-leucine-arginine motif and promote angiostasis by interaction with their common receptor CXCR3 [17]. In tumor microenvironment, in response to specific chemokines, different immune cell subsets recruit and play distinct effects on tumor progression and therapeutic outcomes [18]. Meanwhile, chemokines directly target non-immune cells, including tumor cells and vascular endothelial cells, and function to regulate tumor cell proliferation, cancer stem-like cell properties, cancer invasive and metastasis [18].

Chemokines are up-regulated by a lot of pro-inflammatory cytokines, including TNFα [19]. However, the mechanism for the down-regulation and the termination of chemokine production stimulated by TNFα is not fully understood. In this study, we found that the pre-treatment of human colorectal cancer HCT116 cells with TNFα down-regulated the production of CXCL1, 2, and 8, induced by TNFα re-treatment. TNFα treatment induced the expression of A20. A20 over-expression inhibited TNFα-induced chemokines. A20 inhibition by RNA interference reversed the tolerance of chemokine production induced by TNFα pre-treatment. Meanwhile, we found that TNFα induced chemokines via ERK signal pathway. ERK inhibition decreased chemokine production. Both TNFα pre-treatment and A20 over-expression inhibited ERK signaling. All these results suggested that A20 regulated TNFα-induced chemokine production via inhibition of ERK signaling.

2. Materials and methods

2.1. Cell lines and reagents

Human colorectal cancer HCT116 cells were purchased from ATCC (Manassas, VA, USA). Cells were grown in DMEM containing 10% FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin. Recombinant human TNFα, human CXCL8 ELISA kits were purchased from PeproTech (Rocky Hill, NJ, USA). Rabbit anti-human phosphorylated ERK, JNK, p38 and NF-κB P65 antibodies, rabbit anti-human A20, IκB-α antibodies, and ERK inhibitor U0126 were purchased from Cell Signaling Technology (Beverly, MA, USA). P38 inhibitor SB202190, JNK inhibitor SP600125, and NF-κB inhibitor Bay117082 were purchased from Tocris (Ellisville, MO, USA). A20 mammalian expressing plasmid (EX-K6040-M11) was purchased from Genecopoeia (Rockville, MD, USA). Mouse anti-human TNFR1 antibody was purchase from R&D systems (Minneapolis, MN, USA).

2.2. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from 1 to 2 × 10^6 cells by using of TRIzol (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. mRNA was reverse transcribed with RevertAid (MBI Fermentas, Burlington Ontario, Canada) at 42 °C for 60 min, and the resulting cDNA was subjected to PCR (94 °C for 1 min followed by 20–25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 1 min and an extension for 10 min at 68 °C). The PCR products were separated on 1.0% agarose gels and visualized with GelRed (Biotium). The forward and reverse primer pairs are listed (5′ to 3′) as follows:

A20-F: ATGAGGCCAAAAGGACAGAA
A20-R: ACTGAAAGCATTCGTTGCAG
GAPDH-F, AATCCCATCACCATCTTCCA,
GAPDH-R, CCTGCTTCACCACCTTCTTG;
CXCL1-S: TCACCCCAAGAACATCCAAA
CXCL1-A: TCCTAAGCGATGCTCAAACA
CXCL2-S: GCAGGGAATTCACCTCAAGAA
CXCL2-A: AACACATTAGGCGCAATCCA
CXCL8-S: TTGGCAGCCTTCCTGATTT
CXCL8-A: TCAAAAACTTCTCCACAACCC
TNFR1-S: TTCACCGCTTCAGAAAACCA
TNFR1-A: GGGATAAAAGGCAAAGACCA
TNFR2-S: TGACCAGACAGCTCAGATGTG
TNFR2-A: ACTGCATCCATGCTTGCATT

2.3. Immunoblot

1–2 × 10^6 cells were lysed in 200 ml lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin). The cell lysate was centrifuged at 12,000*g* for 5 min at 4 °C. Proteins were electrophoresed on 10% SDS-PAGE gels, and transferred onto Immobilon P membranes (Millipore, Billerica, MA, USA). The membranes were blocked by incubation in 3% nonfat dry milk at room temperature for 1 h and then incubated with primary antibodies in PBS containing 0.01% Tween 20 at 4 °C overnight. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with SuperSignal chemiluminescent substrate-stable peroxide solution (Pierce Rockford, IL, USA) and BIOMAX-MR film (Eastman Kodak Co., Rochester, NY, USA). When necessary, the membranes were stripped with Restore Western blot stripping buffer (Pierce) and re-probed with antibodies against various cellular proteins.

2.4. Quantitative real time RT-PCR (qRT-PCR)

The qRT-PCR was performed as described by Sun et al. [20]. Briefly, total RNA was isolated and reverse transcribed as described above. The cDNA was amplified by using of TaqMan Universal PCR master mix (Roche Applied Science) and a LightCycle 96 detection system (Roche Applied Science). The amplification of the target genes was normalized by using of the amplification levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The efficiency of the PCR was tested by amplification of the target from serially diluted cDNA generated from the reverse transcription of a stock set of human RNA. The data analysis and calculations were performed using the $2^{-\Delta\Delta CT}$ comparative method, as described by the manufacturer. Gene expression is shown as the fold induction of a gene measured in TNFα-treated samples relative to samples cultured with medium. Same primes were used as described in RT-PCR.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The production of CXCL8 in culture supernatants was detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' standard protocols.

2.6. Plasmid transfection

Cells, cultured in six-well plates, were transfected with 1 μg plasmid containing sequence coding for human A20 protein using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Expression of A20 in the transfected cells was examined by Western blot 48 h after transfection. For stable transfection, G418-resistant cells were selected after incubation with 800 μg/ml G418 for 3 weeks.

2.7. siRNA transfection

siRNA against human A20 (sc-37655) and silencer negative siRNA control (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNA transfection reagent (sc-29528, Santa Cruz, CA, USA) was used to transfect siRNA into HCT116 cells according to the manufacturer's instructions. Briefly, 0.25–1 μg siRNA and

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