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A let-7/Fas double-negative feedback loop regulates human colon carcinoma cells sensitivity to Fas-related apoptosis

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ABSTRACT

Interferon-γ (IFN-γ) is considered essential for the regulation of anti-tumor reactions as it sensitizes Fas-related apoptosis in HT29 cells, but the mechanism is unclear. In the current study, our data demonstrated that IFN-γ stimulation and Fas activation suppressed Dicer processing and let-7 microRNA biogenesis, while let-7 microRNA strongly inhibited Fas expression by directly targeting Fas mRNA. Accordingly, our results indicate that Fas and let-7 microRNAs form a double-negative feedback loop in IFN-γ and Fas induced apoptosis in colon carcinoma cell line HT29, which may be an important synergistic mechanism in anti-tumor immune response. We also found that a let-7 microRNA inhibitor increased Fas expression and sensitized cells to Fas-related apoptosis, which may have future implications in colon carcinoma therapy.

1. Introduction

Apoptosis occurs frequently in epithelial cells located in the gastrointestinal tract, significantly contributing to epithelial cell turnover and the lymphocyte-mediated antitumor response [1]. Studies have demonstrated that epithelial cell apoptosis is regulated by the cell surface receptor Fas. Fas is a member of the tumor necrosis factor receptor family of death receptors and can induce apoptosis in sensitive cells by binding to their specific death ligands, including Fas ligand (FasL), tumor necrosis factor-α (TNF-α) and Fas-specific monoclonal antibody CH11 (mAb CH11) [2,3]. Fas is highly expressed in normal human colon epithelial cells, but its expression becomes progressively decreased during the transition from normal epithelium to adenocarcinoma in approximately 50% of observed cases [4–6]. It is also well documented that most colon carcinoma cells show impaired responses to Fas-related apoptosis. Consequently, from an immunotherapy standpoint, the extent of responsiveness of malignant cells to Fas signals may profoundly influence the overall efficacy of any anti-tumor lymphocyte-mediated response, highlighting the need to explore strategies to sensitize tumor cells to Fas-mediated apoptosis.

Interferon-γ (IFN-γ), an important member of the interferon family that regulates antiviral, antiproliferative, and immunomodulatory responses, has been reported to be involved in the regulation of apoptotic processes. This involvement includes the sensitization of various target cells to Fas-mediated death, such as ovarian cancer cells [7], renal cell carcinoma cells [8] and prostate carcinoma cells [9]. For colon carcinoma, IFN-γ can sensitize human primary colon carcinoma cells to Fas-mediated apoptosis by enhancing Fas expression [10,11]. Previous studies have demonstrated that many genes are involved in the process of IFN-γ-mediated sensitization, including IFN consensus sequence-binding protein and caspase-1 [12]. The underlying mechanism behind how these genes contribute to IFN-γ induction remains unclear; therefore, investigating this molecular mechanism warrants further study.

MicroRNAs are a class of non-coding RNAs that post-transcriptionally regulate protein expression. MiRNA processing is initiated by nuclear RNase III Drosha and completed by cytoplasmic RNase III Dicer [13]. Drosha in a complex with DGCR8/Pasha cleaves a long primary transcript (pri-miRNA) liberating a precursor microRNA (pre-miRNA) with characteristic hairpin structure [14,15]. After its nuclear export, the pre-miRNA is further processed by Dicer into mature miRNA ranging in size from 18–24 nucleotides [16,17]. Recent studies highlighted the importance of microRNAs in cancer cell apoptosis. MicroRNAs miR-21 [18] and miR-17-92 cluster [19] were demonstrated to possess anti-apoptotic function in glioblastoma cells and lung cancer cells, while miR-29 [20] and miR-34 [21] exhibited pro-apoptotic functions by sensitizing cells to apoptosis. Based on previous studies, we hypothesized that certain microRNAs are also involved in the IFN-γ sensitization of human colon carcinoma cells to Fas-mediated apoptosis. Here, we demon-

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strated that Fas expression was post transcriptionally regulated by mciroRNA let-7, while activated Fas influenced Dicer processing of precursor let-7 (pre-let-7). Our results indicate a double-negative feed back mechanism underlying the regulation of Fas protein expression in colon carcinoma cell apoptosis.

2. Materials and methods

2.1. Mice and cell lines

Female athymic nude mice (at 4–6 weeks of age) were purchased from the Transgenic Animal Research Center, Second Military Medical University. The mice were maintained and used in accordance with the institutional guidelines for animal care. The human embryo kidney epithelial cell line HEK293, the human colon adenocarcinoma cell line HT29 and HCT116, the human hepatocellular carcinoma cell lines HepG2 and Huh7 were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). HEK293 cells were maintained in DMEM medium containing 10% FBS (Invitrogen, Carlsbad, CA). HT29 and HCT116 cells were maintained in McCoy's 5A medium with L-glutamine (Invitrogen, Carlsbad, CA) containing 10% FBS. HepG2 and Huh7 cells were maintained in MEM medium with L-glutamine containing 10% FBS.

2.2. Reagents and antibodys

Human rIFN-γ was purchased from R&D Systems (Minneapolis, MN). Anti-Fas activating monoclonal antibody clone CH11 (mAb CH11) was purchased from Millipore Corporate (Billerica, MA). MicroRNA minics and GMR-miR™ microRNA inhibitors were purchased from Genepharma (Shanghai, China).

2.3. RNA preparation, reverse transcription and quantitative real time PCR

Total RNA was prepared from cultured cells using TriZol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. For mature microRNA assays: total RNA (100 ng) was used for cDNA preparation using microRNA specific stem-loop primers (Applied Biosystems (AB), Austin, TX). cDNA was then subjected to Taqman Quantitative Real Time PCR using microRNA specific Taqman primers and PCR master mix (AB, Austin, TX) using StepOne Plus Real time PCR system (AB, Austin, TX). U6RNB was used as endogenous control to calculate fold change using $2^{-\Delta\Delta Ct}$ method with StepOne Software V2.1 (AB, Austin, TX).

For mRNA, the primary transcript of let-7a-1 (pri-let-7a-1) and pre-let-7a-1 assays: 1 μg of total RNA was subjected to DNase treatment (Sigma, St. Louis, MO) followed by cDNA preparation using Oligo dT primer (Fermentas, Glen Burnie, Maryland), dNTPs and MMLV-Reverse transcriptase (Fermentas, Glen Burnie, Maryland). The primers used for PCR were: for Fas: 5′-TTC CCA TCC TCC TGA CCAC-3′ and 5′-CTC GTA AAC CGC TTC CCTC-3′; actin-B: 5′-AGT TGC GTT ACA CCC TTT CTTG-3′ and 5′-GCT GTC ACC TTC ACC GTT CC-3′; pri-let-7a-1: 5′-GAT TCC TTT TCA CCA TTC ACC CTG GAT GTT-3′ and 5′-TTT CTA TCA GAC CGC CTG GAT GCA GAC TTT-3′; pre-let-7a-1: 5′-TGG GAT GAG GTA GTA GGT TC-3′ and 5′-TAG GAA AGA CAG TAG ATT GTA TA-3′.

2.4. DNA constructs and Luciferase assay

For luciferase reporter assays: we cloned the 3′-UTR (1766-2144) of Fas (NM_000043) from human genomic DNA and cloned in pMiR-Report vector (Ambion, Austin, TX) using the Sca I and Hind III (New England Biolabs (Beijing) Ltd, China) site. The following primers were used: 5′-GAG CTC TGA ACA GGC AGG CCA CTT TG-3′ and 5′-AAG CTT CAT TAG GCC ATT AAG ATG AGC ACC-3′. A pMiR-Report construct containing the Fas 3′-UTR with three point mutations in the seed sequence was synthesized with a QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, Palo Alto, CA). These reporters were transfected into HEK293 cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA), and transfection efficiency was detected by co-transfection with the Renilla luciferase vector pRL-CMV (Promega, Madison, WI).

2.5. Measurement of apoptotic cell death

Tumor cells were treated (or untreated) for 24 h with mAb CH11 (1 μg/ml) of either human rIFN-γ (250 U/ml; PeproTech, Rocky Hill, NJ), microRNA mimics (50 nM), or inhibitors (50 nM). Surviving cells were counted using the Cell Counting Kit-8 (Dojindo, Japan). Apoptotic cell death was evaluated by either detecting caspase-3/7-like activity (DEVD-ase activity) using the ApoOne Kit (Promega, USA) or by flow cytometry using the Annexin V-fluorescein isothiocyanate/PI reagent Kit (Nanjing KeyGen Biotech Co., Ltd. China) in accordance with the manufacturers' protocols.

2.6. Cell surface Fas analysis

IFN-γ- (250 U/ml) or mAb CH11-(1 μg/ml) treated and untreated tumor cells were incubated with a biotinylated anti-Fas mAb or an isotype-matched control (Biolegend, San Diego, CA). Cells were then washed, incubated with Streptavidin-PE (Biolegend, San Diego, CA), and analyzed by flow cytometry.

2.7. Western blotting analysis

Proteins were resolved with 10% SDS–polyacrylamide gels and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA). Primary antibodies used were mouse anti-Dicer mAb (Abcam, Cambridge, MA). GAPDH (antibody from Abcam) was used as endogenous control.

2.8. Anti-Fas mAb tumor treatment

HT29 tumors were established ($n = 5$ per treatment group) on female athymic nude mice at 4–6 weeks of age by an intradermal injection of 10^7 HT29 cells (in 50 μL of McCoy's 5A medium) into the hind flanks (day 0). On days 4, 6, and 8, a dose of 20 μg of anti-Fas mAb CH11 was injected intratumorally with 20 μg of let-7 inhibitors, control RNA (in 100 μl PBS) or IFN-γ (1000 U). Tumor sizes were measured twice weekly. Mice were euthanized when tumors exceeded 400 mm^3 in size.

2.9. Statistical analysis

Data are expressed as mean ± SD of experiments performed in triplicate. All figures depicting flow cytometry data represent at least three independent experiments. Statistical comparisons were performed using Student's *t*-test two treatment groups.

3. Results

3.1. Let-7 microRNAs and Fas are inversely expressed in various carcinoma cell lines

Considering the potential contribution of microRNAs in the regulation of Fas, an online search using TargetScan 5.1 was used to find microRNAs predicted to bind to CD95/Fas mRNA, including the let-7/mir-98 family. Playing an important role in post-transcriptional regulation, microRNAs regulate gene expression