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Title:
“A study of Fas-ligand in human nasal benign and malignant neoplasms”

Sheen-Yie Fang, M.D.
Professor
Department of Otolaryngology
College of Medicine
National Cheng Kung University
Tainan, Taiwan
Abstract

Background: Apoptosis mediated through Fas and Fas-L is now recognized as an important mechanism for regulating and limiting this response and is essential in regulating immune function, organ develop and conferring the immune privilege. It was also proved some tumor cells expressed Fas-L.

Fas-L is not only one of three major cytolytic pathways used by cytolytic T cells to kill target cells, but is also a key element in the elimination of activated T cells during the downregulation of the immune response. The pathogenesis of nasal neoplasms, such as polyps, inverted papilloma and carcinoma about their easy recurrence, tendency of malignant change are still unclear. In order to illustrate the possible role of the Fas-L system in the tumorigenesis of human nasal neoplasms, the expression of Fas-L is required to be investigated.

Methods: We collect the fresh specimens form patients who had nasal polyps, inverted papilloma or carcinoma ect. The transcripts and protein level of Fas-L gene are investigated using reverse transcription—polymerase chain reaction and western blotting. Localization of Fas-L is performed by immunohistochemistry, double staining technique. The inferior turbinate mucosa was used as a control. Then we set up the explant culture system to evaluate the effect of steroid or specific antisense or ribozyme. We compare the expression of Fas-L using the above methods between pre and post-treatment. Furthermore, we apply the steroid to the polyp or papilloma patients (in vivo) for 3 months. Then, compare between pre and post-treated expression of Fas-L.

Results & Conclusion:

Fas-L is overexpressed in nasal polyps, inverted papilloma and Squamous cell carcinomas et al. The RT-PCR and western blotting showed the higher level of fas-ligand in the neoplasms. Immunostaining study illustrated the positive cells were at the
epithelial cells, infiltrating lymphocytes and tumor cells. It is feasible that Fas-L will play an important role in the tumorigenesis of these nasal neoplasma. Through the explant tissue culture system (steroid and anti-sense study) and in vivo study, the steroid will downregulate fas-l level in the nasal polyps; however, the Fas-L level will not be changed significantly in the inverted papilloma subjects. In addition, inhibiting Fas-L production and consequently suppressing Fas-L cytotoxicity may be also beneficial and provide as complementary therapy of human nasal neoplasms especially the nasal polyps.
Introduction

Apoptosis mediated through the interaction of Fas and Fas-L is now recognized as an important mechanism not only in the functional organ development but also in pathological changes such as cancer or autoimmune diseases [6,7]. Fas is expressed on a variety of cell types, including lymphocytes [8] and neutrophils [9], whereas Fas-L is expressed predominantly on activated T cells [10], spleen and thymus. Expression of Fas-L in nonlymphoid tissues such as eye, testis, brain may confer the immune privilege of those tissues [11,12]. Fas-L may contribute to the immune privilege of tumors such as hepatoma and melanoma [17]. These tumor cells not only escape Fas-mediated killing by down regulation of the expression of Fas molecules, but also have the ability to actively kill T-lymphocytes by expressing Fas-L protein on the cell surface [18-20].

Nasal polyp, though not a fatal disease for human being, is a common nasal disease with high recurrence. The pathogenesis of nasal polyps is still an enigma. Several findings indicated that infiltration of eosinophil cells [1,2], mast cells [3] or T and B-lymphocytes [4] in tissue of nasal polyps might have influences on or relation to polyp formation. It is believed that etiological factors may include pathogen infection, allergy and immunological disorders. Based on pathological findings, Tos & Mogens have proposed the “epithelium rupture theory” or new gland formation theory” for the polyp formation [5]. They suggested that formation of the tubular gland from the epithelium may occur by down-growth of the basal cells into lamina propria during the early stage of polyp formation. The questions regarding how those down growing cells form gland structure, which followed by cystic degeneration, and how they can evade immune surveillance remain unexplained.

The pathogenesis of inverted papilloma, its easy recurrence and the
tendency of malignant change are still unclear. Some reports proved the HPV infection in IP and correlate to malignant transformation. In order to illustrate the possible role of the Fas/Fas-L system in the tumorigenesis of human IP or associated with viral infection, the expression of Fas-L is required to be investigated. Other nasal malignant neoplasms also require these informations regarding Fas-L system to get more detaile in their pathogenesis.

It has been definitly proven that topical ateroid treament can reduce polyp size and associated nasal symptoms. Glucocorticosteroids are the only type of drug having a proven effect on symptoma and signs of nasal polyposis. Topically applied steroid in controlled clinical trials. They reduce the size of polyps and prevent, in part, their recurrence. Thus, we want to investigate whether Fas-L involves the pathway of which steroid using for nasal polyps therapy. We constructed a ribozyme specific for Fas-L mRNA to regulate the Fas-L expression. Thus, applying these ribozyme in the explant culture system, to evaluate the dynamic changes of Fas-L, that will be helpful in understanding the pathogenetic role of Fas-L in these neoplasms.
Materials & Methods

(1) RT-PCR

Total RNA was prepared by the RNeasy Total RNA kit according to the manufacture's instruction (Qiagen, Hilden, Germany). Total RNA was converted to cDNA utilizing oligo-dT as a primer and StrataScript™ H-reverse transcriptase (Stragene, CA, USA). The reaction mixture was incubated at 37°C for 1 hr, and then heated to 90°C for 5 minutes to denature the reverse transcriptase. The generated cDNA was subjected to PCR amplification on a DNA Thermal Cycler (Hybaid Omnigene, Middlesex, UK) as described previously by Yang et al. [13]. PCR products were fractionated by agarose electrophoresis, stained with ethedium bromide and visualized under UV light.

(2) Western blotting

Tissues were minced by frozen section machine. Cells were extracted with the buffer containing 1% triton X-100, 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mg/ml aprotinin and 50 μg/ml PMSF. Proteins were separated in a 12.5% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. The proteins bounded on the membrane were probed with rabbit IgG anti-Fas-L polyclonal antibody (N-20, Santa Cruz Biotechnology, CA) at 4 °C for 16 hr. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Dako, CA, USA) was used as secondary probe and the immune complexes were made visible by fluorography with enhanced chemiluminescence (ECL) detection kit (Amersham International PLC, UK).

(3) Immunohistochemistry

The localization of Fas-L was detected by immunostaining using an
IgG anti-Fas-L polyclonal antibody. Cryosection of nasal samples were transferred onto glass slide coated with poly-L-lysine fixed, heated with microwave and fixed with 4% paraformaldehyde in phosphate buffered saline. Fixed cells were permeabilized at room temperature for 15 min with 0.1% Triton-X-100 in PBS and subsequently blocked by incubation with 0.5% bovine serum albumin. Cells were then hybridized with anti-Fas-L antibody for 3 hr followed by a goat anti-rabbit IgG conjugated with horseradish peroxidase. After extensive washing with PBS, cells were mounted on a glass slide over a drop of p-phenylenediamine/glycerol, examined by epifluorescence microscopy and photographed.

Other specific special stainings for epithelial cells, lymphocytes were performed to identified specific positive cells.

(4) Explant culture system

Tissue Handling

Human inferior turbinates, polyps et. al. were obtained from patients undergoing elective surgery. Within 20 min of surgical excision specimens were placed in L15 medium supplemented with penicillin (100 U/ml), streptomycin (100μg/ml), and amphotericin B (0.5μg/ml) for transport to the laboratory.

Human Nasal Mucosal Explant Culture

Fresh nasal specimen was cut into 3×3 mm fragments. Pairs of fragments were placed on 5×10 mm Gelfoam pads in 35-mm petri dishes. The mucosa cultures (80 to 100 mg wet tissue/dish) were maintained in 2 ml of CMRL 1066 medium containing penicillin (100 U/ml), streptomycin (100μg/ml), and amphotericin B (0.5μg/ml) in a controlled atmosphere chamber gassed with 45% O₂, 50% N₂, and 5% CO₂, and incubated at 37°C. After 24 h of initial culture, medium was replaced with fresh medium containing 400 KIU/ml aprotinin to inhibit the
proteases capable of degrading ET-1. Experiments were performed after an additional 24 h of culture. The tissue form each patient was used for one or two experiments. Study of explants with and without budesonide (100 μg/ml) were performed for 3 days. Another study of explants with and without specific ribozyme were performed, too. Then these tissues were processed for RT-PCR, Western blotting and Immunohistochemistry study.

(5) Synthesis of Specific Ribozyme

(a) Construction of pEGFP-N1-rA & pEGFP-N1-rB plasmids

Two ribozymes (rA, rB) are constructed to cut specifically the Fas-L mRNA. Ribozyme A targets specifically the sequence locating to extracellular domain of both human and rat Fas-L gene. Ribozyme B recognizes specifically the transmembrane domain of only rat Fas-L. The ribozyme A is as follow and was synthesized by genemed synthesis, Inc, USA.

Ribozyme A:

5' ATG AAT TCC CGG AAG TAC TGA TGA GTC GTG ATA
CGA CGA CGA AAC TTT GGA TCC CGA 3'
5' TCG GGA TCC AAA GTT TCG TCG TAT CAC GAC TCA
TCA GTA CTT CCG GGA ATT CAT 3'

The synthetic oligonucleotides of ribozyme were suspended in H2O of 1 μg/l. After annealing, set A was digested with EcoRI/BamHI and was digested with EcoRI/Smal. The digested DNA products were purified by phenol/chloroform extraction followed ethanol precipitation. The vector plasmids, pEGFP-N1, were treated with the corresponding enzymes for cloning. DNA ligation was performed as standard method described by Sambrook et al.
(b) Transformation

The E.coli strain DH5-α was transformed with ligation products and selected with kanamycin at a concentration of 100μg/ml. Plasmid DNA was purified with Wizard Plus SV Minipreps DNA Purification System kit according to manufacture’s description. Correct plasmid constructs were confirmed by restriction enzyme mapping and DNA sequencing.

(c) Amplification of plasmids

Recombinant plasmid DNA for transtection were isolated utilizing CsCl density gradient according to the standard method described by Sambrook, et al. Plasmid DNA were suspended in TE buffer at a concentratiion of 500μg/ml and stored at 4°C.

(6) Checking the Ribozyme

(a) Cell Culture

U-118MG and U-373MG are human glioblastoma cell lines described as grade III astrocytoma-glioblastomas and obtained form American Type Culture Collection. Cells were grown in Dulbecco’s modified Eagle,s medium (DMEM) containing glucose 1g/liter, L-glutamine (2mM), 100μl/ml streptomycin, 100 units/ml penicillin and 20% fetal bovine serum. Cells were grown as monolayer to 80% confluency and transferred 1:2 every 3-4 days.

C6

C6 is a rat glial tumor-derived cell line and is kindly provided by Drs. Tsieo-Gung-Lin & Dr. Tzeng Ra-Tung. Cells are grown in DMEM containing glucose 4.5g/liter, L-glutamine (4mM), 100 μl/ml streptomycin, 100 units/ml penicillin and 10% fetal bovine serum.
(b) Transfection

Twenty-four hours prior transfection, $1 \times 10^5$ cells/well were seeded to a well of six well plate. After 24 hour culture, cells were transfected with 5μg of DNA/well (dissolved in TE) using the lipotransfectamin (Superfect; Qiagen, USA) some transfection technique. Forty-eight hours after transfection, cells were harvested for analysis.

(c) FACScan

After 48 hr-transformation, cells were harvested and fixed in 100μl of 4% formaldehyde/PBS. After 1 hour incubation on ice, cells were permeabilized with 200μl buffer containing 0.1% Triton X-100/0.1% sodium citrate for 10 minutes and wash by PBS 3 time. Following stain with primary antibody (1/1000x, rabbit IgG anti human Fas-L, cross-reaction with mouse and rat Fas-L) and secondary antibody (1/1000x, mouse anti rabbit IgG with PE conjugated). The Fas-L protein is detected by FACScan machine.

(d) Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The transcripts of Fas-L were analyzed by semi-quantitative RT-PCR. Total RNA of cells is isolated with Rneasy Mini Kit and converted to cDNA with reverse transcriptase MMLV according to manufacture’s description
Results:
Both nasal polyps and nasal turbinates revealed a similar level of the transcripts of fas-L gene. However, an enhanced expression of the fas-L protein was shown in protein extracts of nasal polyps using western blotting. Fas-L was localized predominantly to the epithelial cells of submucosal glands and the downward growing epithelium of nasal polyps.

Fas-L is overexpressed in nasal polyps, inverted papilloma and squamous cell carcinoma et al. The RT-PCR and western blotting showed the higher level of fas-ligand in the neoplasms. Immunostaining study illustrated the positive cells were at the epithelial cells, infiltrating lymphocytes and tumor cells. It is feasible that Fas-L will play an important role in the tumorigenesis of these nasal neoplasia. Through the explant tissue culture system (steroid and anti-sense study) and in vivo study, the steroid will downregulate fas-l level in the nasal polyps; however, the Fas-L level will not be changed significantly in the inverted papilloma subjects. In addition, inhibiting Fas-L production and consequently suppressing Fas-L cytotoxicity may be also beneficial and provide as complementary therapy of human nasal neoplasms especially the nasal polyps.
Conclusion: The fas-L is significantly enhanced in the epithelial cells of nasal polyps, compared to nasal turbinates. The fas-L also shows a higher level in the nasal neoplasms such as inverted papilloma, SCC etc. The level can be downregulated by steroid (in vivo and in vitro). These results suggest that the fas/fas-L system may play an important role in the pathogenesis of human nasal polyps and neoplasms.
References


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