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ORIGINAL ARTICLE

ROLE OF IL-23 IN CROHN'S DISEASE AND ULCERATIVE COLITIS AND OTHER PREMALIGNANT DISEASE, IT'S THERAPEUTICS APPROACH

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ABSTRACT: Crohn's Disease and Ulcerative colitis collectively known as inflammatory bowel disease IBD is chronic uncontrolled inflammation characterized by intense mucosal recruitment of activated leukocytes. Here we have developed an in vitro model for IBD using colon cancer cell line HCT-116. Colon Cancer cell line HCT-116 expressed elevated IL-23 levels when induced with LPS isolated from various bacterial strains. In this in-vitro model of IBD, IL-23 was playing a key role in inflammation cascade. Action of anti-inflammatory drug Sulfasalizine was studied and was found to block the up regulation of IL-23.

KEYWORDS: IBD (Inflammatory Bowel Disease), HCT-116, IL-23 (Interleukin-23), Sulfasalizine, LPS (Lipopolysaccharide).

INTRODUCTION: Inflammation can be defined as a series of non-specific defense mechanism of body, in which cells and different mediators respond to tissue injury.[1] Inflammation, the response of tissue to injury, is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines. In the sub-acute/chronic phase it is characterized by the development of specific humoral and cellular immune responses to the pathogen(s) present at the site of tissue injury.[2]

Most cytokines involved in the inflammation processes are multifunctional. They are pleiotropic molecules that elicit their effects locally or systemically in an autocrine or paracrine manner.[2]

Cytokines are involved in extensive networks that involve synergistic (Proinflammatory cytokines) as well as antagonistic interactions (anti-inflammatory cytokines) and exhibit both negative and positive regulatory effects on various target cells.[3]

Sometimes immune cells fail to distinguish among the foreign and its native cells, as a result the process of inflammation is targeted towards its own cells, the condition is termed as Auto inflammation.

One such condition is Inflammatory Bowel Disease in which the gut micro biota if through any pathway gets interacted with its own underlying immune cells; the process of inflammation towards these microorganisms begins there by leading to the destruction of tissue as such

Inflammatory bowel disease (IBD) refers to two chronic diseases that cause inflammation of the intestines: Ulcerative colitis (UC) and Crohn's disease (CD). The hallmark of inflammatory bowel disease is chronic uncontrolled inflammation characterized by intense mucosal recruitment of activated leukocytes.[4] Although the diseases have some features in common, there are some important differences.

Over the past 15 years wide variety of candidate genes have been studied for IBD. Significant linkages have been reported on chromosomes 1,3,6,7,12,14,16 and 19. Detailed mapping of chromosome 16 resulted in identification of the gene responsible, at least in part for this linkage.[5]

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This gene encodes a cytoplasmic protein designated nucleotide-binding oligomerization domain 2 NOD2 also known as caspase activation and recruitment domain CARD15.[4] This is a polymorphic gene the product of which is involved in the innate immune system. It is the first gene to be clearly associated with IBD, and >60 mutations have been recognized, 3 of which have been linked to development of CD. The mechanism whereby defects in the NOD2 gene lead to the development of IBD remains unclear.

The NOD2 gene is expressed mainly in monocyte/macrophage cell lines, where it has a role in host signaling pathway. Reasonable data suggest that mucosa of the patients with established Crohn's disease is dominated by CD4+ lymphocytes with a type 1 helper-T-cell (Th1) phenotype, characterized by production of IFN-γ, IL-2 IL-8 and IL-23. In contrast the mucosa in patients with ulcerative colitis may be dominated by CD4+ lymphocytes with an atypical type 2 helper-T-cell (Th2) phenotype, characterized by the production of transforming growth factor β (TGF-β) and IL-5.[5]

MATERIALS AND METHODS: HCT-116 cells are also adherent colonic epithelial cells obtained from the patients of colorectal adenocarcinoma but with different cellular products mainly carcinoembryonic antigen (CEA); transforming growth factor beta binding protein; mucin. The cell line used in the present study was purchased from American Type Culture collection (ATCC), Manassas VA 20108, USA.

Reagents used in the study like DMEM Powder, Bovine serum albumin (BSA), DMSO, Sodium bi carbonate, Trizma, EDTA and others were obtained from Sigma (Sigma Aldrich co. St. Louis MO USA).

ELISA Kit for both IL-23 was quantitated from the cell supernatants using DuoSet ELISA Development KITS. Sandwich ELISAs are sensitive enzyme immunoassays that measure soluble levels of proteins in biological samples. R&D SYSTEM provides Complete ELISA kits that offer accurate and reproducible results with no development time Duo Set kits contain the basic components required to develop an immunoassay to measure natural or recombinant proteins. All these were obtained from R&D Systems, Inc 614 McKinley Place NE, Minneapolis, USA.

METHODOLOGY: In our study HCT-116 cells were cultured with a medium consisting of DMEM, 10%FETAL BOVINE SERUM (FBS) and 1% penicillin –streptomycin antibiotic solution. The cells were incubated at 37°C under a humidified atmosphere of 5% CO2 in air.

Seeding: Around 80% confluency stage, the cells were sub cultured. Medium contained in the flask was completely discarded using a sterile disposable pipette. Cells were washed with 1X PBS so as to remove the dead cells and the cell debris. Cells were treated with trypsin-EDTA solution (0.25%W/V) and kept at 37°C in the CO2 incubator for 2-5 mins. The cells were detached from the substratum, as monitored under the phase contrast microscope. Once the cells were completely detached, immediately excess amount of complete medium was added to the cells.

This is because a prolonged exposure of cells with the trypsin-EDTA solution is known to cause cell death.

The serum contained in the complete medium acts as an inhibitor of trypsin. Flushing gently with a pipette tip mixed the contents of the flask. From this cell suspension, a sample was subjected to counting using a hemocytometer.

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The desired no. of cells was selected by diluting it with complete medium. Seed the cell suspension diluted with the medium in the respective plate. (12, 24, or 96 well plate) according to the requirement of the experiment. Incubate the respective plates over night at 37°C in the CO2 incubator prior to any treatment, for ensuring proper growth and spreading of cells.

Treatment with LPS and the Drug Addition: Specific cell line was selected based on the experiment and the cells were allowed to get confluent. Post confluent cells were seeded in 96 well plate as described above, with a concentration of 30,000-40,000 cells/well. Plate was then left at CO2 incubator for overnight incubation.

As designed for the experiment 1) if the cells are to be pre-incubated with drug followed by LPS treatment, the cells in the plate were incubated with drug in different concentrations. Based on the solubility of the drug if soluble in water was directly diluted with medium and applied to the cells or if insoluble was the diluted in DMSO and the applied. 3 hours later of drug incubation cells were treated with LPS to get inflamed. LPS treatment was optimized using different strains of bacteria and maximum efficient strain to cause inflammation was chosen for the experiment.

Based on the volume of the cell suspension in the well LPS was introduced respectively. Followed this plate containing cells were incubated in CO2 incubator for overnight. Next day supernatant from each well was collected by centrifuging plate at 1500 rpm for 15 min. Supernatant from the plate was collected in sterile eppendorfs and was stored as samples for ELISA at -80°C.

The plate with the adherent cells was then undertaken for the MTT assay for measuring the cell viability. To assess the maximum tolerance of cells for the given concentration of drug MTT assay was performed. ELISA was performed as per the user manual provided with the R&D Duo kit system. Observations of ELISA are depicted graphically as follows with interpretations.

OBSERVATIONS AND RESULTS:

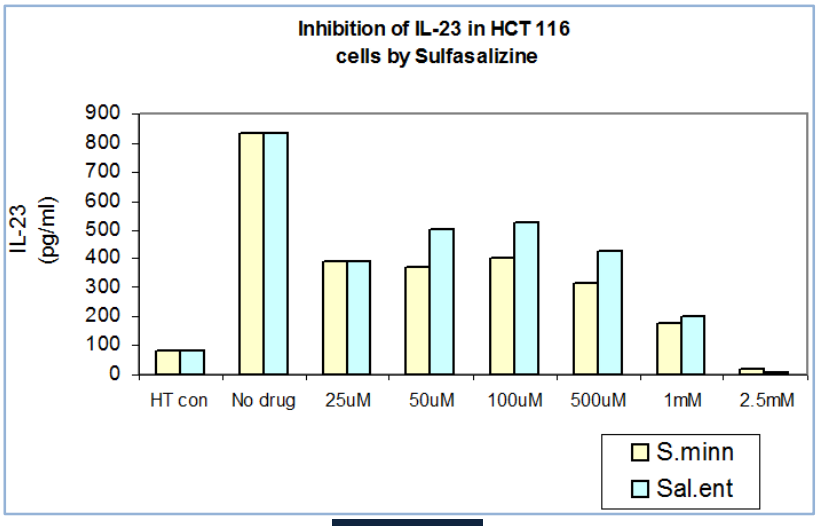

Figure 1

The above graph shows induction of IL-23 in HCT-116 cells by LPS isolated from the strains of Salmonella Minnesota and Salmonella Enteritidis. First plot in the graph shows the control group. Second plot shows the induction of IL-23 by LPS strain. From third plot onwards different

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concentration of drug is provided to down regulate the IL-8 production and almost 100% inhibition is seen around 1mm–2.5mm drug concentration

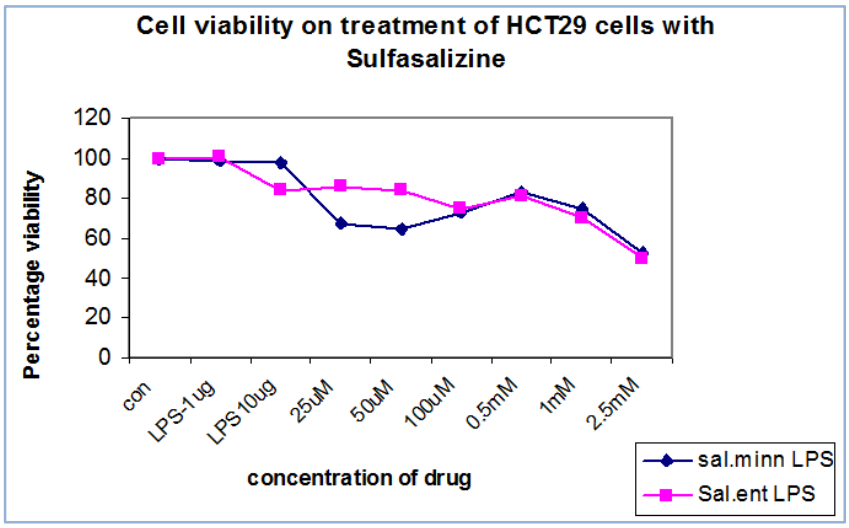

Figure 2

Cell viability for the above drug concentration was assayed simultaneously using MTT assay and it was found that drug can be safely used till a concentration of around 1mM and thereafter number of cell death was found to be more.

DISCUSSION: The Inflammatory Bowel Disease (IBD) is a chronic auto inflammatory disease where intestinal epithelial cells (IEC) gets differentiated as cancer cells when induced by some inflammatory agents. Normally in-vivo these epithelial cells remain separated from underlying immune cells by a membrane lining. In most of the cases of IBD the underlying immune cells comes in direct contact with micro-biota of intestine resulting in the process of inflammation,[6] thereby leading to uncontrolled tissue destruction.[7]

The process of inflammation could be studied with the help of certain tumour markers. In preparing the in-vitro model for IBD several cytokines were used as prominent markers whose upregulation determined the stages of inflammation.[2]

In-vitro model for IBD could be developed by using colon cancer cell line like HCT-116, CaCo-2, SW-620 and others which show some inflammatory responses. In our experiment we have used HCT-116 cell which expressed various proteins indicating inflammatory responses.[8]

Development of inflammatory conditions in HCT-116 cell line could be achieved through various ways. In several studies the bacterial DNA or bacteria as a whole were confirmed to express inflammatory responses.[9,10]

The idea to introduce Lipopolysaccharide (LPS), a product of gram negative bacterial cell wall as an inflammatory agent is a new approach of our research laboratory for this study since no study in this regard has been made so far.

In normal intestinal flora (in-vivo) numbers of bacterial strains exist but few of them have been found to be pathogenic in nature. LPS was isolated from different bacterial strains and were introduced in HCT-116 cells. In the selection of LPS we used strains of E.coli B-4 (Present in

abundance in intestine) to elicit infection. It was found that E.coli B-4 did not induce much inflammatory conditions in HCT-116 cells. Then LPS was isolated from different strains of bacteria like Salmonella Minnesota (S.Minn.), Salmonella Enteritidis (S.Ent.) and Pseudomonas to study the inflammatory reactions. It was observed that S.Minn and S.Ent were most potent inflammatory agents.

After developing inflammatory conditions in colon cancer cell line HCT-116 with LPS isolated from bacterial strains of S. Minn and S. Ent we then checked inflammatory responses with cytokines as a marker.[11] Cytokines are categorized under pro-inflammatory and anti-inflammatory in nature.

In our present study we selected Interleukin-23 (IL-23) cytokine for observing the effect of LPS. LPS induction expressed different levels of IL-23 with respect to each bacterial strain.[12] Upregulation of IL-23 cytokine (Proinflammatory) confirmed the inflammatory conditions in given cell line.

Further in our study to check the inflammation process developed, the effect of certain anti-inflammatory drugs was studied in the same.[13] The popularly used drug for IBD like Sulfasalizine at different concentration was introduced to inhibit the upregulated cytokine IL-23.[14] Complete or above 70% inhibition of upregulated IL-23 with minimum concentration of Sulfasalizine was regarded as efficiency of drug to mimic the in-vivo model of IBD.[15] (Fig. 1).

Dose response curve (DRC) was set up for each given concentration of drug with respect to bacterial strain and cell line to study the cytotoxic effect and most effective concentration with least cytotoxic effect was analyzed (Fig. 2).

CONCLUSION: HCT-116 cells expressed different levels of inflammatory processes for in-vitro model of inflammatory bowel disease. Lipopolysaccharide (LPS) isolated from strains of Salmonella Enteritidis and Salmonella Minnesota of gram-negative bacteria induced inflammation in the monolayer and co-culture system of inflammation. IL-23 levels were highly expressed in the process of inflammation. Sulfasalizine at different concentration was found effective in treatment of inflammatory bowel disease and other. Premalignant disease so that there is no chance of any Conversion of premalignant to malignant disease.

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