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Theaflavins retard human breast cancer cell migration by inhibiting NF-κB via p53-ROS cross-talk

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ABSTRACT

The present study demonstrates that theaflavins exploit p53 to impede metastasis in human breast cancer cells. Our data suggest that p53-dependent reactive oxygen species (ROS) induce p53-phosphorylation via p38MAPK in a feedback loop to inhibit IκBα-phosphorylation and NF-κB/p65 nuclear translocation, thereby down-regulating the metastatic proteins metalloproteinase (MMP)-2 and MMP-9. When wild-type p53-expressing MCF-7 cells are transfected with p53 short-interfering RNA, or treated with a pharmacological inhibitor of ROS, theaflavins fail to inhibit NF-κB-mediated cell migration. On the other hand, NF-κB over-expression bestows MCF-7 cells with resistance to the anti-migratory effect of theaflavins. These results indicate that inhibition of NF-κB via p53–ROS crosstalk is a pre-requisite for theaflavins to accomplish the anti-migratory effect in breast cancer cells.

Structured summary:
MINT-7295816: *p53* (uniprotkb:P04637) *physically interacts* (MI:0915) with *IKK beta* (uniprotkb:O14920) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Metastasis is the most frequent cause of death in patients with advanced breast cancer and still poses a challenge to successful cancer therapeutics, inhibiting which may provide more effective treatment for patients with advanced breast malignancy.

Recently, use of phytochemicals to impede cancer metastasis by addressing multiple targets that regulate the migratory capacity of cancer cells, has gained immense importance. In this regard, theaflavins, the bioactive flavonoids of black tea, have been demonstrated to induce apoptosis and to inhibit tumor cell invasion in a variety of cancer cells [1,2]. However, the underlying mechanisms responsible for the anti-migratory effects of theaflavins in breast cancer are largely unknown.

It is acknowledged that in addition to its pro-apoptotic functions, the tumor suppressor protein p53 contributes to the regulation of migration [3] and p53 mutation triggers the metastatic potential in breast cancers [3]. Convincing evidence suggests that p53 executes its function via reactive oxygen species (ROS) generation [4]. It has been reported that ROS inhibit metastasis of human breast cancer [5] and removal of oxygen radicals by anti-oxidant drugs impedes tumor-suppressing effect of p53 [4]. In fact, ROS function as signaling molecules in upstream by triggering p53-activation through post-transcriptional modification like phosphorylation [6] and in the down-stream by leading to apoptosis after being generated via p53-induced redox active genes [7,8]. Therefore, approaches employing therapeutic agents that generate ROS in tumor cells could significantly enhance the therapeutic gain.

Increasing evidence suggests that NF-κB-associated pathways are deregulated in numerous malignancies, including breast cancer [9,10]. The transcription factor NF-κB has been observed to promote endothelial cell motility while RNAi suppression of NF-κB inhibited bladder cancer cell migration [11]. Inhibition of NF-κB activity significantly reduced proliferation and invasion of Hep3B cells as well as down-regulated the expression of invasion-related molecules including metalloproteinase (MMP)-2 and MMP-9 [12]. Recent studies demonstrate the significance of p53 in regulating NF-κB-activation through different mechanisms [13]. Data also illustrate that sustained oxidative stress may

Abbreviations: DCFH-DA, dichlorofluorescin diacetate; MMP, metalloproteinase; NAC, N-acetyl-cysteine; ROS, reactive oxygen species; siRNA, short-interfering RNA; Wt, wild-type

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inactivate the proteosome and subsequently inhibit NF-κB-activation by impeding the degradation of IκBα [14]. Although an inter-relationship among enhancement of p53, increment in ROS and inhibition in NF-κB that led to cancer cell apoptosis, has been demonstrated [15], to date hardly any information exits about the cross-talk, if any, between p53, ROS and NF-κB in relation to retardation of human breast cancer cell migration by plant polyphenols, theaflavins.

The present study describes that theaflavins retarded human breast cancer cell migration by inhibiting NF-κB/p65 via p53–ROS feed back loop. In fact, p53-dependent up-regulation of proline oxidase and down-regulation of manganese superoxide dismutase (MnSOD) resulted in generation of ROS that in turn phosphorylated and activated p53 in a redox-regulatory loop via p38MAPK to block NF-κB/p65 nuclear translocation by inhibiting IκBα-phosphorylation. Inhibition of NF-κB in turn caused down-regulation of pro-migratory enzymes MMP-2 and MMP-9. While transfection of p53-short-interfering RNA (siRNA) protected wild-type p53 (Wt-p53)-expressing MCF-7 cells from the anti-migratory effect of theaflavins, over-expression of Wt-p53 sensitized mutant p53-expressing MDA-MB-231 cells to theaflavin-insult, thereby confirming the contribution of p53. Furthermore, inhibition of ROS or transient over-expression of NF-κB rendered MCF-7 cells resistant to theaflavin-insult. Altogether, these results elucidate an important role for theaflavins in suppressing breast cancer metastasis by activating p53/ROS/p38MAPK positive feed back loop that hindered NF-κB activation to finally inhibit pro-migratory enzymes MMP-2 and MMP-9.

2. Materials and methods

2.1. Cell culture and treatments

Human breast carcinoma cells (MCF-7, ZR-75-1, MDA-MB-231) were obtained from NCCS, India. The MDAH041 post-crisis skin fibroblast cell line was derived from a patient with Li-Fraumeni syndrome. Cells were routinely maintained in complete DMEM in a humidified CO_2 incubator [1].

Cells were treated with 10 μg/ml theaflavins (Sigma, USA) for 12 h, unless stated otherwise, to examine its anti-migratory effect. To assess the role of ROS and p38MAPK, MCF-7 cells were treated with ROS scavenger N-acetyl-cysteine (NAC) (Sigma, USA) for 1 h or p38MAPK inhibitor (SB 203580, 10 μM) (Calbiochem) for 90 min prior to incubation with theaflavins.

2.2. Wound healing assay

Cell migration was determined by means of unidirectional wound healing assay as described [16]. Briefly, cells were grown to confluency in 12 well plates after which a sterile blade was used to scratch the monolayer of cells to form a unidirectional wound. Migration was quantitated by a semi-automated, computer-assisted procedure by a person blinded with respect to the experimental treatment. The data from triplicate wells were calculated as the means ± S.E.M., the migration rate of control cells was taken as 100% and healing rate of other plates were compared with control cells.

2.3. Assessment of ROS

Cells were incubated for 20 min at 37 °C in the dark with 10 μM of dichlorofluorescin diacetate (DCFH-DA). DCF fluorescence was measured flowcytometrically (Beckton Dickinson FACScan) and subjected to analysis using Cell Quest 3.2 (Beckton Dickinson) software. The probe was excited at 488 nm and emission was measured through a 530 nm band-pass filter. For confocal microscopy a Leica fluorescent microscope DM 900 was used to visualize the images of cells treated with theaflavins and stained with DCFH-DA. Digital images were captured with a cool (−25 °C) charged coupled device (CCD) camera (Princeton Instruments) controlled with the MetaMorph software.

2.4. Western blot analysis and coimmunoprecipitation

To assess the involvement of different proteins, whole cell lysates as well as cytosolic and nuclear fractions were prepared and ran on SDS–PAGE gels, transferred to nitrocellulose membranes, as described previously [17,18], and probed with specific antibodies, e.g., anti-p53, p65 (RelA), IκBα, phospho-IκBα (p-IkBα), IKKβ, phospho-Ser15-p53 (p-Ser15-p53), MnSOD, proline oxidase, MMP-2 and MMP-9, procured from Santa Cruz. For the determination of direct interaction between p-Ser15-p53 and p65 or IKKβ, p-Ser15-p53 immunocomplexes from cytosol were purified using anti-p-Ser15-p53 antibody and protein A-Sepharose beads. The immunopurified proteins were immunoblotted with anti-p65 or anti-IKKβ. The protein of interest was visualized by chemi-luminescence. Equivalent protein loading in nuclear and cytosolic fractions was verified using anti-histone H1 and anti-α-actin antibodies (Santa Cruz), respectively.

2.5. Plasmids, siRNA and transfections

Wt-p53 was transfected in MDA-MB-231 cells through an adenoviral vector expression system expressing Wt-p53 under the control of cytomegalovirus promoter (Clontech). The cDNA encoding p65 subunit of human NF-κB was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The resulting p65-pcDNA3.1 plasmids (4 μg each/million cells) were introduced separately into MCF-7 cells using LipofectAmine 2000 (Invitrogen). Stably expressing clones were isolated by limiting dilution and selection with G418 sulphate (Cellgro) at a concentration of 1 μg/ml and cells surviving this treatment were cloned and screened by Western blot analysis with specific antibodies. MCF-7 cells were transfected separately with p53-/control-ds-siRNA and p65-/control-ds-siRNA (Santa Cruz) using LipofectAmine. The levels of p53 and p65 expression in selected clones were estimated by Western blotting.

2.6. Statistics

Values are shown as standard error of mean (S.E.M.), except otherwise indicated. Data were analyzed and when appropriate, significance of the differences between mean values was determined by Student's *t*-test. Results were considered significant at $P < 0.05$.

3. Results

3.1. Theaflavins inhibit migration of mammary epithelial carcinoma cells expressing Wt-p53

Theaflavins have been found to successfully inhibit migration of Wt-p53 containing MCF-7 cells in a dose-dependent manner (Fig. 1A). However, since doses above 12.5 μg/ml of theaflavins are apoptogenic in breast cancer cells, as evident from our previous studies [1], further experiments were restricted to sub-apoptotic dose of 10 μg/ml. Interestingly, under these conditions, theaflavins failed to furnish any significant effect in mutant p53-containing MDA-MB-231 cells (Fig. 1B). These results led us to hypothesize that anti-migratory effects of theaflavins might be favoured in Wt-p53-expressing mammary epithelial carcinoma cells.