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The role of STAT-6 as a key transcription regulator in HeLa cell death induced by IFN-γ/TNF-α co-immobilized on nanoparticles

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ABSTRACT

Based on the fact that the transcription of STAT-1 plus its Serine 727 and Tyrosine 701 phosphorylation is not the pre-requisite for the cell death signal transduction in the IFN-γ signaling pathway induced by co-immobilized IFN-γ/TNF-α, we investigate both *in vitro* and *in vivo* the key transcription regulators to promote the signal transduction of HeLa cells. It is found that IFN-γ R2 is the important death signal receptor in the HeLa cell death by RNA interference. Checking the expression of the whole transcription (STAT) protein family reveals that STAT-6 is highly expressed in comparison with the other STAT proteins. The gene silence of IFN-γ R2 leads to the down-regulation of STAT-6 and phosphorylation-STAT-6 (p-STAT-6) expressions. The successful gene silence of STAT-6 results in the reduction of HeLa cell programmed death and the expression of several important key factors related to programmed cell death (p53, Bcl-2, and Bax). More importantly, our *in vivo* experiments by injecting nanoparticle drug carriers with the co-immobilized IFN-γ/TNF-α into nude mice model confirm the high expression of STAT-6 and p-STAT-6. It is thus concluded that, in response to IFN-γ, the co-immobilized IFN-γ/TNF-α unusually promotes the activation of STAT-6 rather than STAT-1, resulting in the enhanced cell programmed death in HeLa. The present work reveals the gene-level molecular mechanism of IFN-γ/TNF-α co-immobilized on biomaterials as a potentially effective therapy against cancer cells.

1. Introduction

Among the drugs to induce high antitumor efficacy *in vitro* and *in vivo*, biomaterials, which offer advantages of delayed release and long action have been the trend of the development of drugs. Along this line, in an earlier work we synthesized a polymeric drug model based on co-immobilized interferons-γ plus tumor necrosis factor alpha (IFN-γ/TNF-α) onto the polymeric substrates, followed by a series of *in vitro* investigations on the significant anti-cancer effects of this drug model [1]. Moreover, we reported our observation on the upstream cell membrane surface receptor with which the co-immobilized IFN-γ/TNF-α binds, and then addressed how the co-immobilized IFN-γ/TNF-α promotes the signal transduction of HeLa cells. Our results suggest that the death signal transduction is mainly realized via the IFN-γ signaling pathway rather than the TNF-α one. In addition, it was identified that the transcription of STAT-1 (STAT-s stands for signal transducers and activators of transcription-s) is not the pre-requisite for inducing the cell death signal transduction, which is an unusual outcome and deserves for substantial investigations. A direct argument is that the co-immobilized IFN-γ/TNF-α may promote the activation of some other key markers in response to IFN-γ [2]. A clarification of the underlying cell biology mechanism with which this drug model works becomes critical not only in pushing this drug model itself into next step for potential applications, but more importantly for updating our knowledge on the IFN-γ/TNF-α as popular platform on which the anti-cancer cell biology is focused.

Indeed, IFN-γ is a cytokine mainly produced by NK and Th1 cells, which plays a critical role in the host defense against pathogens and tumors. IFN-γ exerts its biological activities by interacting with a specific cell surface receptor (IFN-γ R) made of two chains: IFN-γ R1 chain and IFN-γ R2 chain. The best characterized transduction pathway induced by IFN-γ is the Jak/STAT-1 pathway, which inhibits the proliferation of many types of cells [3–5]. As well known,

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STAT-1 is phosphorylated at two sites (tyrosine 701 (Y701), and serine 727 (S727)) following the exposure to IFN-γ. IFN-γ receptor/JAK complex directly mediates Y701 phosphorylation, whereas S727 phosphorylation occurs via a separate pathway involving Ca (2+)/calmodulin-dependent kinase II. Following the phosphorylation, STAT-1 forms homodimers that translocate to the nucleus and bind to the IFN-γ-activated site (GAS) in the presence of the promoters of various IFN-γ-inducible genes, such as IFN-regulatory factor-1 (IRF-1) and ICAM-1 [6–8].

While the above functionalities of STAT-1 are well established, actually STAT protein family has seven components (STAT-s: s = 1, 2, 3, 4, 5a, 5b, and 6) that mediate signal transduction from extracellular to target genes. Among the STAT-s, the activation of STAT-6 is almost limited to the cytokines IL-4 and IL-13. The activation mechanism of STAT-6 appears to be similar to that of other STAT-s. The binding of IL-4 and IL-13 to their receptors provokes the activation of tyrosine kinases. As a result, tyrosine residues within the common phosphorylated IL-4R alpha chain. Specific tyrosines can thus recruit STAT-6 to the receptor complex where it is also phosphorylated. After that, STAT-6 leaves the receptor, dimerizes, and then migrates to the nucleus where it binds to GAS elements in the promoter of inducible genes [9–14]. In addition, the STAT-6 activation is tightly regulated at a number of molecular levels. The Janus (JAK) and Src families of tyrosine kinases regulate the earliest events that lead to STAT-6 phosphorylation [15]. The inhibitory mechanisms involve phosphatases that dephosphorylate STAT-6 and also involve suppressor of cytokine signaling (SOCS) that blocks the kinase activity associated with the receptor [16]. In literature, the STAT-6 has been implicated in the development of allergic diseases and tumors. Definitely, an understanding of the molecular mechanisms involved in the regulation of not only STAT-6 is of great interest for the developing specific biomaterials for treatments of these diseases [17–24].

Indeed, in our earlier work, it was revealed that the role of STAT-1 in the IFN signaling pathway is insignificant, which is different from earlier works without engaging specific biomaterials (co-immobilized IFN-γ/TNF-α onto polymeric substrates) [2]. This finding motivates us to search for an alternative in this STAT family, which is significantly expressed in the cell death signal transduction. Given the known fact that specific biomaterials on which the IFN-γ/TNF-α molecules are chemically bonded via various approaches are engaged, it is not exceptional that the IFN-γ/TNF-α molecular structures may be modulated on one hand, and on the other hand the engaged biomaterials themselves may play some roles. Therefore, the present work, not only as a simple extension to earlier work, would be a substantial step towards understanding of the underlying mechanisms of high anti-cervical cancer functionality of the co-immobilized IFN-γ/TNF-α biomaterials.

Here we pay our major attention to the RNA interference technology (lentiviral shRNA infection and analysis) and gene microarray analysis, so that the key transcription regulator to promote the signal transduction of HeLa cells induced by these biomaterials can be revealed in the gene level. Surprisingly, both the *in vitro* and *in vivo* data reveal the important role of the STAT-6 in the IFN signaling pathway. Our study thus demonstrates a model, with which the subsequent investigation of the role and activation molecular mechanisms of STAT-6 induced by the co-immobilized IFN-γ/TNF-α biomaterials can be explored. Moreover, since inappropriate regulation of specific STAT-s occurs with surprisingly high frequency in a wide variety of human cancers [25,26], an understanding of the activation mechanisms of transcription regulator STAT-6 may lead to the development of biomaterials based on interrupting key steps in this pathway. Also, the immobilization of growth factors is attractive because it enables cell behaviors such as growth, differentiation, and death to be regulated through signal transduction [27]. However, as far as we know, no such work focusing on the transcription regulating of the biomaterials has been available.

2. Materials and methods

In this section, we highlight the main procedure to synthesize the co-immobilized IFN-γ/TNF-α biomaterials and characterizations. The cell biology and molecular biology investigation on the effects of the biomaterials, both *in vitro* and *in vivo*, will be performed. The main part is devoted to the *in vitro* experiments, while the *in vivo* experiments are designed for a demonstration of the unveiled effects in the *in vitro* ones. All the descriptions unless stated specifically are assigned to the *in vitro*.

2.1. Synthesis and co-immobilization of AzPhIFN-γ plus AzPhTNF-α

The preparations of photoactive or IFN-γ (Sigma) and the co-immobilized IFN-γ/TNF-α were separately described previously [1,2,28,29]. All the treatments were carried out in the dark.

2.2. Cell culture and MTT assay

The HeLa cells obtained from the Sun Yat-Sen University were sub-cultured on the plastic tissue culture dishes in RPMI1640 medium supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Subsequently, the HeLa cells were seeded at 1×10^5 cells/ml into the 24-well PSt culture plate which were co-immobilized by IFN-γ plus TNF-α in a previous procedure.

After the 24 h treatment, the HeLa cells were transferred to the 96-well microtiter plates with PBS. Then, MTT (0.5/mg/ml) was added, followed by incubation at 37 °C for 2 h in a CO_2 incubator. After a brief centrifugation, the supernatants were carefully removed and DMSO was added. After those insoluble crystals were completely dissolved, the Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) was used to measure the absorbance value of formazan and a strong absorbance at 540 nm was detected. The related results are summarized in Fig. 1.

2.3. Lentiviral shRNA infection and analysis

To uncover the function and role of IFN-γ R2 and STAT-6, we used the lentiviral shRNA infection to obtain the effect of gene silence. The construction of RNA interference vector and screening of design target for IFN-γ R2 and STAT-6 were performed by JiKai Gene Company, Shanghai, China. In the trial experiments, the lentiviral shRNA for different target sequences (LV-1, LV-2, LV-3, LV-4) were used for different treatments. For IFN-γ R2, we used LV-1, LV-2, LV-3, and LV-4, respectively. However, for STAT-6, we used LV-1, LV-2, LV-3 plus LV-4 to enhance the transfection efficiency which was determined based on the green fluorescent protein. After the HeLa cells were transfected with RNA interference oligonucleotides or control oligonucleotides, the efficiency of the RNA interference-mediated IFN-γ R2 and STAT-6 protein suppression was determined by western blot analyses (using the antibodies to IFN-γ R2 and STAT-6 obtained from Beijing Zhongshan Golden Biotechnology Co., China, respectively). The related results are summarized in Figs. 1 and 5.

2.4. Gene microarray analysis

To find the key transcription regulator, the gene microarray analysis was performed. The HeLa and HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ cells were seeded at 1×10^5 cells/ml into the 24-well PSt culture plate, which were co-immobilized by TNF-α plus IFN-γ. Here HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ represents the HeLa with knockdowned IFN-γ R2 gene. At the same time, the HeLa cells were induced by free IFN-γ/TNF-α in another 24-well PSt culture plate for a comparison purpose. We assign those HeLa cells treated by the co-immobilized IFN-γ/TNF-α and free IFN-γ/TNF-α as the co-immobilized HeLa group and free HeLa group, respectively. In the meanwhile, we assign the HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ cells treated by the co-immobilized IFN-γ/TNF-α as the co-immobilized HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ group. After the 24 h treatment, the HeLa and HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ cells were harvested, washed with cold PBS, centrifuged, and added with the RNA extraction Kit, stored in −80 °C. Then the samples were submitted to Beijing Biological Technology Limited Corp. for microarray experiments following the procedure described in Ref. [30].

After the microarray experiments, both the up-regulative genes in chip-*a* (the co-immobilized group compared with the free group) and the down-regulative genes in chip-*b* (the co-immobilized HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ group compared with the co-immobilized HeLa group) were picked out. The STAT family including seven related genes was selected. Their plots, expression bitmaps, relevant information, and clustering diagram, are shown in Fig. 2.

2.5. Immunofluorescence analysis

For the immunofluorescence analysis of protein expression, the co-immobilized HeLa or HeLa$^{\text{IFN-}\gamma\text{R2shRNA}}$ group (IFN-γ plus TNF-α = 10 ng/well plus 10 ng/well) were treated respectively for 24 h, and then washed twice with the PBS, fixed with 10% paraformaldehyde for 20 min at room temperature, permeabilized using 1% Triton-X 100 for 15 min, blocked with the PBS containing 1% BSA and 10% cattle serum for