A natural aryl hydrocarbon receptor ligand, indirubin, causes the p21(waf1/cip1) up-regulation cooperated with tumor necrosis factor-alpha

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Introduction

Indirubin, isolated from human urine, is a natural ligand of the aryl hydrocarbon receptor (AhR) and has 50 times stronger AhR activating activity when compared to 2,3,7,8-tetrachrolodibenzo-*p*-dioxin in the yeast assay for AhR ligand activity.[1] We previously reported that cell-cycle arrest and cell differentiation occurred when human lymphoma cell line ML-1 was co-treated with indirubin and tumor necrosis factor-alpha (TNF-a) in DIOXIN2003. Further investigation showed that cell cycle arrest occurred in the G1 phase and that p53-independent up-regulation of the cyclin-dependent kinase inhibitor, $p21^{(waf1/cip1)}$, is involved with this phenomenon (unpublished data). In the p21 promoter region, many transcription factor binding sites are included, and many p53-independent p21 activation pathways exist.[2] To determine which transcription factors and which p21 activation pathways are mainly involved with this up-regulation is of importance. In this study, we first constructed a firefly luciferase vector containing p21 promoter regions of various lengths. After which, the region that mainly relates to the up-regulation of p21 was evaluated and the candidate transcriptional factor binding sites that take part in cell-cycle arrest were explored using a reporter gene assay.

Materials and Methods

Materials Indirubin was synthesized as described previously and kindly provided by Dr. Saeki (Nagoya City University, Nagoya, Japan).[3] Vectors and the Dual-Glo™ luciferase assay system were purchased from Promega (Wisconsin, USA). Oligonucleotides were purchased from SIGMA genosys (Hokkaido, Japan). Restriction enzymes, T4 polynucleotide kinase, T4 Ligase, TaKaRa Ex Taq® R-PCR Version 2.1 and DNA ligation kit ver.2.1 were purchased from TaKaRa Bio Inc. (Shiga, Japan). All other chemicals and reagents were purchased from Wako Chemical (Osaka, Japan).

Cell culture The human breast cancer cell line MCF-7 was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37^{o}C in a humidified air incubator supplemented with 5% CO_2.

Cell viability assay Cells were seeded at 1×10^4 cells per well, in 96-well plates and incubated overnight. Fresh DMEM was used and contained chemicals in various concentrations. For solvent contrast, cells were treated with DMSO (0.1% v/v). Cell viabilities were determined every 24 hours using the crystal violet method. Method details were as follows: culture media was washed and cells were stained with crystal violet by incubation with a staining solution containing 0.1% crystal violet in 10% methanol for 10 min at room temperature and rinsed gently three times in distilled water. The dye was extracted with methanol and absorbance was read at 590 nm by a micro plate reader.

Plasmids The p21 promoter DNA fragment was synthesized in three separate parts (pGLwaf459; -443~+16bp, pGLwaf1587; -1571~+16bp, and pGLwaf2621; -2605~+16bp). For pGLwaf459, commercially available

oligonucleotides were phosphorylated by T4 polynucleotide kinase and bound by T4 ligase. Synthesized DNA fragments were amplified by PCR using the following oligonucleotides: 5'-CAGTGAGCTCATTAATGTCATCCTCCTGATCT-3' and 5'-TACTAGATCTAGGAACTGACTTCGG-3'. The others were generated by PCR amplification using genomic DNA extracted from ML-1 cells as the template. The oligonucleotides used as primer were as follows: 5'-CAGTGAGCTCAGGTCCTGAGGCTG TGCCGTGG-3', 5'-TCGAATTAATACATAAAAATTCAT-3' (for pGLwaf1587), 5'-CAGTGAGCTCAATTCCTCTGAAAGCTGACT-3', and 5'-GCACAGCCTCAGGACCCCACTCTA-3' (for pGLwaf2621). All DNA fragments were cloned into the Sac I andBgl IIsites of the pGL3-basic vector (which includes the firefly luciferase coding region) by using DNA ligation kit ver. 2.1. In the case of pGLwaf459, the following oligonucleotides were used as linkers; 5'-CAGTCGTACGTAGTAGC-3' and 5'- TAGCTACTACGTACGACTGAGCT-3'.

Transfection and luciferase assay Cells were seeded at 80-90% confluence in 10-cm dishes on the day before transfection. Cells were co-transfected with *Renilla* luciferase expressing vector, pRL-TK, as an internal control, using lipofectamine 2000 reagent according to the manufacturer's instructions. Six hours later, the transfection reagent was removed and cells were harvested into 12-well plates. Twenty-four hours after transfection, cells were treated with chemicals and DMSO. Following 12 hours of incubation, cells were collected and dissolved in 75 ml of DMEM without serum and phenol red, and firefly and *Renilla* luciferase activities were measured using the Dual-Glo™ luciferase assay system according to manufacturer's instructions. Each assay was practiced three times.

Results and Discussion

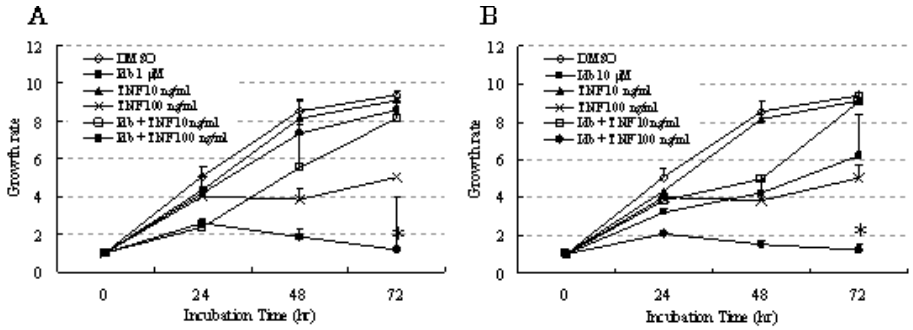

Fig. 1 MCF-7 was seeded at 1×10^4 cells per well, in 96-well plates and after overnight incubation, cells were treated with tested chemicals or DMSO (0.1% v/v). Cell viabilities were determined every 24 hours using crystal violet. When they were co-treated with indirubin (**A**: 1 μM and **B**: 10 μM) and TNF-a (100 ng/ml)? these chemicals synergistically arrested cell growth similarly.
* Significantly different from DMSO ($p < 0.05$)

Cell viability To examine whether cell growth arrest in the ML-1 cell line was also observed in the MCF-7 cell line, cell viabilities after exposure to indirubin and TNF-a at various concentrations were determined. When cells were exposed to indirubin or TNF-a only, results indicated that cell growth arrest was not observed at any concentration. However, when cells were co-treated with indirubin (≥1 mM) and TNF-a (100ng/ml), these chemicals synergistically caused arrested cell growth regardless of the concentration of indirubin as long as it was above 1 mM (Fig. 1). The effective concentration was three orders of magnitude greater than that of ML-1. Considering that indirubin is rapidly metabolized by CYP1A1 induced by itself, the potency of induction of CYP1A1 might be different between MCF-7 and ML-1.[4] Further research such as measuring the difference of the metabolic rate of indirubin and TNF-a in MCF-7 and ML-1 should be required.

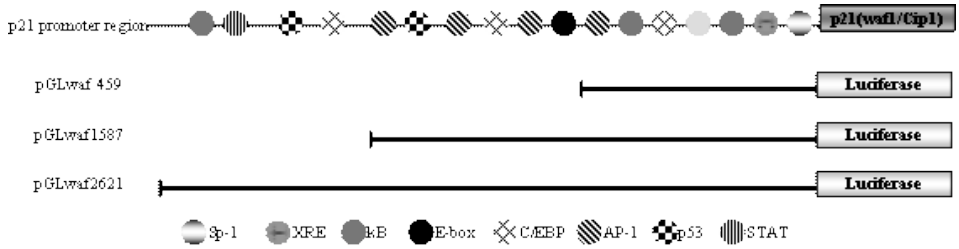

Fig. 2 The vector constructs and major transcription factor binding sites are represented. The p21 promoter region was cloned into pGL3-basic which expresses the firefly luciferase gene. Each vector contained several important binding sites for activating p21 gene.

Luciferase activity Previous research has revealed that the peak of induction of p21 mRNA was 8 hours later from the point when stimulation started (unpublished data), therefore, we decided upon an exposure time of 12 hours after considering the time for protein synthesis in this study. Firstly, changes in luciferase activities with various concentrations of indirubin were determined using pGLwaf2621. As shown in Fig. 2A, in the case of co-treatment of indirubin and TNF-a, the luciferase activities were significantly different at all concentrations ($p < 0.01$). In addition, the luciferase activity intensity was changed in a concentration-dependent manner after 12 hours of exposure. The same results were also observed in pGLwaf459 and pGLwaf1587 (data not shown). In order to obtain more clear data, the exposure concentration of indirubin was decided to be 10 mM in this study. Fig. 2B shows the pattern of luciferase induction for three vectors we investigated. In all vectors, the luciferase activity was higher when cells were exposed to both indirubin and TNF-a. However the differences in luciferase induction patterns as related to chemical exposure were not observed. Therefore, it seems that the transcription factor binding sites included in pGLwaf459 are mainly associated with this p21 induction as caused by combined exposure of indirubin and TNF-a. In the region -443~+16, many transcription factor binding sites exist, for example AP-1, kB, XRE, and Sp-1, and they play important roles in p53-independent p21 regulation. It is natural to regard that these two transcription factor binding sites, AhR, which is activated by indirubin and associates with XRE, and NF-kB which is activated by TNF-a and associates with kB, caused this p21 up-regulation. Indeed, several studies have reported the relationship between XRE and kB in genes which have both binding sites within their promoter region.[5, 6] However, with respect to p21, it was reported that one natural ligand of AhR, 3, 3'-diindolylmethane, activates the Sp-1 sites of p21 and promotes p21 protein synthesis and therefore that AhR has a relationship to Sp-1.[7, 8] Considering these facts, further research is required for revealing the mechanism of p21 up-regulation combined with the effects of indirubin and TNF-a using shorter reporter vectors other than pGLwaf459.

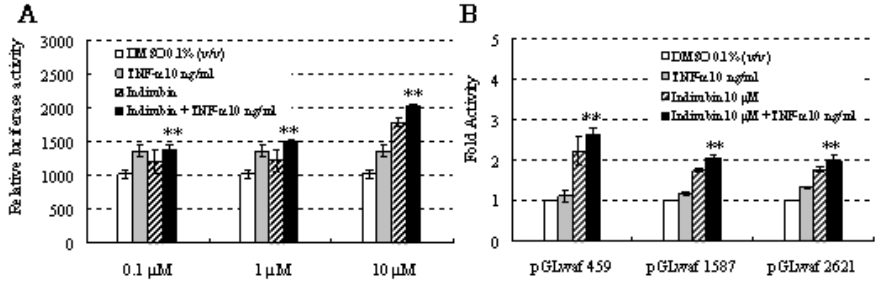

Fig. 3 The results of luciferase assay. **A**: The change in luciferase activities with various concentrations of indirubin was determined using pGLwaf2621. MCF-7 cells were co-transfected with pGLwaf2621 and pRL-TK. Twenty-four hours later, cells were treated with chemicals or DMSO (0.1% v/v). After 12 hours of incubation, the firefly and *Renilla* luciferase activity were measured. The intensity of luciferase induction changed in concentration-dependent manner. **B**: The pattern of luciferase induction related to the plasmids was determined. The method of measurement was the same as **A**. The bars show the average data of the fold activity of luciferase induction versus DMSO (0.1% v/v). The pattern of luciferase induction did not changed among the three vectors, pGLwaf459, pGLwaf1587, and pGLwaf2621. The extent of induction change was not very different among vectors. ** shows that the data were significantly different from DMSO ($p<0.01$).

In this research, we constructed a reporter vector that included that codes for the longest p21 promoter region (-2605~+16) and our experiment revealed that the specific region where ligand binding occurs is located in the region from -443 to +16 of the p21 promoter. However, the exact sequence which functions as the ligand binding site is still

unclear, therefore, further research to reveal the specific binding site is required. For example, it may be appropriate to use the luciferase assay with a shorter vector than that used in this research, or to use the electro mobility shift assay.

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