Up-regulation of the expression of major histocompatibility complex class I antigens by plasmid DNA transfection in non-hematopoietic cells

Joo-Hung Park[a,*], Sun-Hee Chang[c], Min-Chul Kim[c], Sung-Hye Shin[c], Hyun-Joo Youn[d], Jin-Kyoo Kim[b], Yong-Suk Jang[e], Chul-Woo Kim[f]

[a] *Department of Biology, Changwon National University, Changwon, Kyungnam 641-773, South Korea*
[b] *Department of Microbiology, Changwon National University, Changwon, Kyungnam 641-773, South Korea*
[c] *CMRC, Presbyterian Medical Center, Chonju, Chonbuk 560-250, South Korea*
[d] *Department of Microbiology, Inje University, Inje, Kyungnam 621-749, South Korea*
[e] *Faculty of Biological Sciences and The Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561-756, South Korea*
[f] *Department of Pathology, Seoul National University Medical School, Seoul 110-799, South Korea*

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Abstract The effect of DNA on the surface expression of major histocompatibility (MHC) class I antigens was examined in non-hematopoietic tumor cell lines. Transfection with plasmid DNA via liposome or electroporation significantly increased the surface expression of MHC class I molecules in a transient manner. Northern blot analysis showed that levels of MHC class I mRNA were increased by DNA transfection, probably via transcriptional activation. In contrast, the expression of the MHC class II and β-actin genes was not affected, suggesting that the up-regulation of MHC class I expression by plasmid DNA works in a gene-specific manner.

Key words: Plasmid; Major histocompatibility; Transfection; Up-regulation; mRNA

1. Introduction

Major histocompatibility (MHC) class I molecules expressed at the cell surface of virtually all somatic nucleated cells are composed of a polymorphic heavy chain non-covalently associated with β_2-microglobulin, and an antigenic peptide derived from proteins degraded in the cytosol and transported into the endoplasmic reticulum (for review, see [1]). Surface levels of MHC class I molecules on antigen presenting cells are critical to induce an appropriate immune response. There is plenty of evidence that up- or down-regulation of MHC class I expression could lead to immunomodulations. Many types of human cancer are known to be deficient of or low in MHC class I expression compared to their normal counterparts [2] and many tumorigenic cells lacking MHC class I molecules are rejected in vivo following MHC class I gene transfection [3]. In contrast, aberrant increases of MHC class I levels could contribute to the occurrence of many pathological conditions including hypomyelination [4] and ankylosing enthesopathy [5].

The expression of MHC class I molecules is regulated mainly at the transcriptional level [6], and *cis*-acting regulatory sequences that are binding sites for transcription factors including NF-κB, retinoid X receptor and CIITA were shown to be operational in the expression of the MHC class I genes [7]. MHC class I expression can be up-regulated by cytokines including IFN-α, IFN-γ and TNF-α [8,9], and by viral infection [10]. In addition, it was recently reported that the surface expression of MHC class I antigens on dendritic cells, which can capture antigens in peripheral tissues and migrate to secondary lymphoid organs where they acquire the ability to stimulate naive T cells, was increased by infection with bacteria like *Mycobacterium tuberculosis* and *Streptococcus gordonii* [11,12] partly via increased stability of MHC class I protein [11]. However, the factors responsible for the effect remain to be determined.

Immunological characteristics of DNA, generally viewed as immunologically inert, have been extensively studied over the past few years [13]. Bacterial DNA, but not vertebrate DNA, induced IFN (α,β,γ) secretion, activated NK cell activity, and inhibited tumor growth [14]. Bacterial DNA also induced B cells to secrete IL-6 and IgM [15], and spleen cells to secrete IL-12 and TNF-α [16]. It was previously reported that the immunomodulatory functions of bacterial DNA were ascribed to either unmethylated CpG motifs flanked by two 5′ purines and two 3′ pyrimidines [17] or a palindromic sequence AACGTT flanked by strings of 5′ and 3′ dG residues in 30-mer oligonucleotides [18]. However, the molecular mechanisms of the immunomodulatory effects of bacterial DNA remain largely unknown.

In this study, we have examined if bacteria-derived DNA can modulate the expression of MHC class I antigens. Here we show that levels of MHC class I antigens on the cell surface of non-hematopoietic tumor cells are up-regulated by liposome- or electroporation-mediated transfection with plasmid DNA, but not by simple addition of the DNA to cultures, in part through the increase of steady state level of MHC class I mRNA. The stability of MHC class I mRNA molecules is not changed by plasmid DNA transfection, suggesting that the increased steady state level of MHC class I mRNA results from transcriptional activation. In contrast, the expression of the MHC class II and β-actin genes is not affected.

2. Materials and methods

2.1. Cell lines, cell culture conditions, and monoclonal antibody

The MCA-102 and MCA-207 cell lines, methylcholanthrene-induced fibrosarcomas, and the CT-26 cell line, a murine colon carcinoma cell line, have been described previously [9,19], and were maintained in DMEM (Gibco-BRL, Gaithersburg, MD, USA) with 10% FBS, supplemented with antibiotics and glutamine. Hybridoma cell

*Corresponding author. Fax: (82) (551) 79-7440.
E-mail: parkjh@sarim.changwon.ac.kr

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lines secreting mAbs 28-8-6s, 34-1-2s and M5/114.15.2 were purchased from ATCC (Rockville, MD, USA).

2.2. Liposome-mediated transfection

Liposome (lipofectin, Gibco-BRL, Gaithersburg, MD, USA) mediated transfection was carried out as recommended by the supplier. Cells were seeded at a density of 2×10^4 cells/well in a 24-well plate and incubated for 24 h in a CO_2 incubator. Cells were washed twice with serum- and antibiotic-free DMEM and incubated for 24 h in the presence of DNA-liposome complex in 0.5 ml of serum- and antibiotic-free DMEM. Cells were washed twice in PBS, fed with DMEM supplemented with 10% FBS and antibiotics, and incubated for an additional 24 h. Cells were harvested for flow cytometry analysis of MHC class I expression. For DNA transfection without liposome, all the procedures were the same except that liposome was deleted. LPS purchased from Sigma (St. Louis, MO, USA) was dissolved in PBS.

2.3. Electroporation

Electroporation was performed as previously described [20]. Adherent cells were first resuspended at a concentration of 1×10^6 cells/0.8 ml in DMEM containing 10% FBS at room temperature. DNA was added to the cell suspension, incubated at room temperature for 10 min, electroporated (Bio-Rad, Hercules, CA, USA; 960 microfarads, 350 mV), and incubated at room temperature for 10 min.

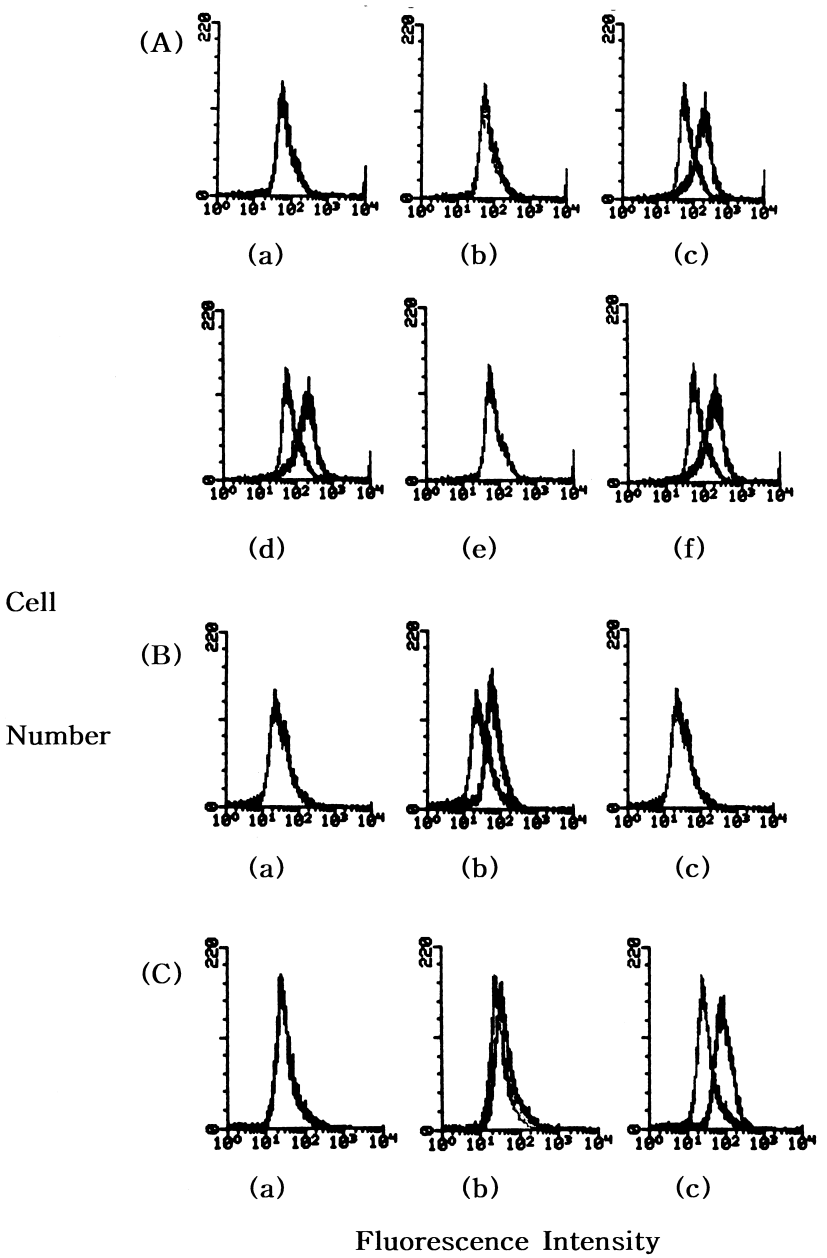

Fig. 1. Increase of MHC class I expression by transfection with plasmid DNA. After transfection, cells were cultured for 48 h, and harvested for flow cytometry analysis of MHC class I expression (H-2K^bD^b). For a better comparison, the control histogram is shown superimposed. A: MCA-102 cells were transfected either by liposome with 1 μg of different plasmid DNAs (b–d) or by electroporation in the presence of 10 μg of pcDNA3.1 DNA (e,f). a: No DNA and no liposome; b: liposome only; c: 1 μg pcDNA3.1 plus liposome; d: 1 μg pBR322 plus liposome; e: mock transfection; f: 10 μg of pcDNA3.1 DNA. B: MCA-102 cells were transfected with pcDNA3.1 pre-treated with DNAse I via liposome. a: Liposome alone; b: 1 μg pcDNA3.1 plus liposome; c: 1 μg pcDNA3.1 pre-treated with DNAse I plus liposome. C: MCA-102 cells were transfected with different concentrations of pcDNA3.1 in the presence of liposome. a: 100 pg pcDNA3.1; b: 10 ng pcDNA3.1; c: 1 μg pcDNA3.1.

The electroporated cells were transferred to 5 ml of DMEM containing 10% FBS, incubated for 48 h in a CO_2 incubator, and harvested for flow cytometry analysis of MHC I expression.

2.4. Flow cytometry analysis of MHC class I expression

Flow cytometry analysis was carried out by indirect fluorescence using a FACSorter (Becton Dickinson, Mountain View, CA, USA). Harvested cells (1.6×10^5) were washed in PBS containing 2% FBS and 0.03% NaN_3, incubated with primary antibodies (28-8-6s for H-2 K^bD^b; 34-1-2s for H-2 K^dD^d; M5/114.15.2 for H-2 I-$A^{b,d,q}$ and I-$E^{d,k}$) appropriately diluted with PBS containing 0.5% BSA for 5 min at 4°C, and washed twice in PBS containing 2% FBS and 0.03% NaN_3. Cell pellets were resuspended in PBS containing 0.5% BSA and further incubated in the presence of FITC-conjugated goat anti-mouse or anti-rat IgG antibody (Sigma) for 5 min at 4°C. After being washed in PBS containing 2% FBS and 0.03% NaN_3 twice, cells were resuspended in PBS containing 0.5% paraformaldehyde, 0.25% BSA and 0.03% NaN_3, and subjected to FACS analysis.

2.5. DNA preparation

A large scale preparation of plasmid DNA (pBR322; pMAL-c2, New England Biolabs, Beverly, MA, USA; pcDNA3.1, Invitrogen, San Diego, CA, USA) from *Escherichia coli* cells (DH5-α, Gibco-BRL) was carried out basically according to the alkaline lysis method [21], and further purified by Qiagen-tip (Qiagen, Hilden, Germany) to remove RNA, proteins, carbohydrates, and metabolites. Toxic and mutagenic substances such as phenol, chloroform, and ethidium bromide were not used. The LPS content of the plasmid DNA was determined by the *Limulus* amebocyte lysate assay (Bio-Whittaker, Walkersville, MD, USA) and the LPS content of all the DNA preparations was less than 5 ng/μg DNA.

2.6. DNase I treatment of DNA

For DNase I treatment of DNA, 1 mg of DNA was incubated in 1 ml of 0.1 M Tris-HCl pH 8.0/5 mM $MgCl_2$ containing 1000 U of DNase I (Promega, Madison, WI, USA) at 37°C for 5 h, boiled for 10 min to inactivate DNase I and then rapidly cooled on ice, as previously described [14]. The degradation of DNA was confirmed on 1% agarose gel electrophoresis.

2.7. RNA preparation and Northern blot analysis

RNA preparation and Northern blot analysis were performed as previously described [20]. The murine α-actin probe was from ATCC (Rockville, MA, USA) and the MHC class I K^d probe was prepared by RT-PCR using a pair of primers based on the sequence [22]. The PCR products were confirmed to correspond to their original sequence by DNA sequencing.

3. Results

To determine if prokaryotic DNA can modulate the surface expression of MHC class I antigens, MCA-102 cells, a low expressor of MHC class I H-$2K^bD^b$ molecules, were incubated with various concentrations of pcDNA3.1, an eucaryotic expression vector. We used plasmid DNA instead of bacterial DNA as immunomodulators, because (i) both plasmid and bacterial DNAs share for immunostimulatory functions essential features such as unmethylated CpG dinucleotides present in multiple copies [23], (ii) plasmid DNA is structually less complex than bacterial DNA, and (iii) it is easy to handle and prepare. A simple addition of various amounts of pcDNA3.1 from 0 to 50 μg and a subsequent incubation for 48 h were not effective in the modulation of the surface expression of MHC class I molecules (data not shown). Intracellular transport of extracellular DNA into mammalian cells could be facilitated by liposome [24] or electroporation [25]. Therefore, we examined if efficient intracellular transport of pcDNA3.1 may have modulatory effects on MHC class I expression. MCA-102 cells were analyzed for MHC class I expression 48 h after transfection with pcDNA3.1 complexed with liposome. Untreated MCA-102 cells showed minimal staining with anti-H-$2K^bD^b$ antibodies with a mean fluorescence of 50 (Fig. 1A,a). Liposome treatment alone had little, if any, modulatory effect on MHC class I expression (Fig. 1A,b). However, 1 μg of pcDNA3.1 complexed with liposome significantly enhanced the surface level of MHC class I proteins to mean fluorescence value of 200 (Fig. 1A,c). Other types of plasmid DNAs including pBR322, a subcloning vector (Fig. 1A,d), and pMAL-c2, a procaryotic expression vector (data not shown), prepared from *E. coli* cells were also

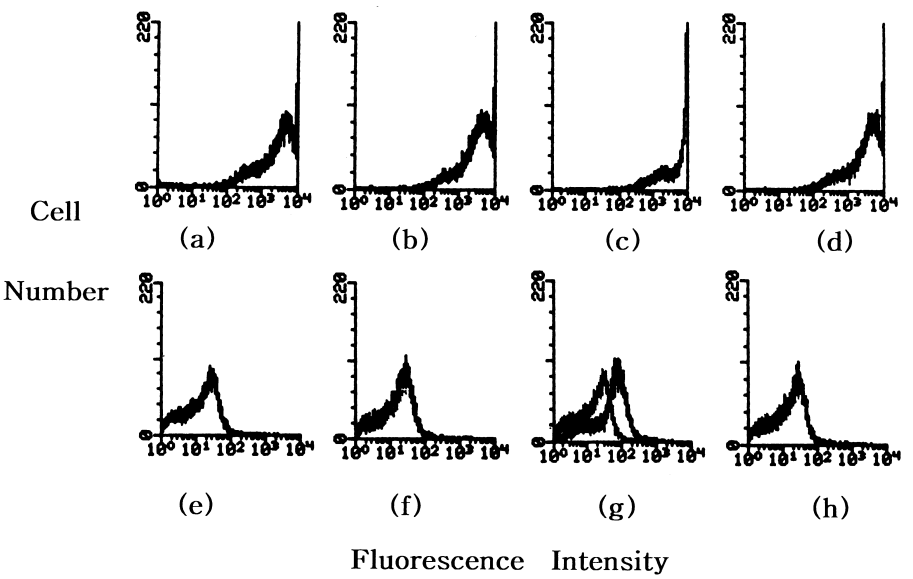

Fig. 2. Effects of pcDNA3.1 on MHC class I expression in CT-26 cells expressing H-$2K^dD^d$ antigens. After transfection, cells were cultured for 48 h, and harvested for flow cytometric analysis of MHC class I expression (H-$2K^dD^d$). a, e: No DNA and no liposome; b, f: liposome alone; c, g: 1 μg pcDNA3.1 plus liposome; d, h: 1 μg pcDNA3.1 pre-treated with DNase I plus liposome. Surface expression of MHC class I antigens in CT-26 cells (a–d) was analyzed under the same instrument conditions set for MCA-102 cells. To improve resolution among samples (a–d) the intensity scale was shifted to the left and analyzed (e–h). The control histogram (e) is shown superimposed.

capable of up-regulating the surface expression of MHC class I molecules as effectively as pcDNA3.1. When transfection was performed by electroporation, surface levels of MHC class I molecules were also increased 3–4-fold by the addition of plasmid DNA (Fig. 1A,e,f). And pre-treatment of pCDNA3.1 with DNase I reverted the effect of the DNA (Fig. 1B).

The plasmid DNAs used in these studies were contaminated with small amounts of LPS (less than 5 ng/μg DNA) whose function on the regulation of MHC class I expression is controversial [26,27]. In our system, no detectable effect of LPS (up to 10 μg) on the expression of MHC class I antigens was observed (data not shown).

We next determined the effective range of DNA dosage in modulating MHC class I expression. Levels of MHC class I antigens increased as the concentration of DNA increased (Fig. 1C), reaching the maximum level at 1 μg. No further increase was observed with more than 1 μg up to 100 μg (data not shown). Less than 10 ng DNA was not effective (Fig. 1C,a).

Kinetic analyses of the expression of MHC class I antigens by liposome-mediated plasmid DNA transfection showed that surface levels of MHC class I expression in MCA-102 cells were transiently increased, reaching a maximum level 2 days after transfection (data not shown).

The up-regulatory effects of plasmid DNA on MHC class I expression shown in MCA-102 cells were also observed with two other tumor cell lines, MCA-207 cells which express comparable levels of MHC class I H-2K^{b}D^b molecules (data not shown), and CT-26 cells which express high levels of H-2K^{d}D^d antigens (Fig. 2). Given all the above results, it is likely that plasmid DNA up-regulates the surface expression of MHC class I antigens when efficiently transfected into different kinds of cells.

To get some insight into molecular mechanisms underlying the increased surface expression of MHC class I antigens by plasmid DNA, Northern blot analysis was performed. Northern blot analysis with 20 μg of total RNA revealed that levels of MHC class I mRNA were increased about 3-fold by plasmid DNA transfection (Fig. 3). The enhancing effect of DNA was abolished by pre-treatment with DNase I (Fig. 3, lane 4). No change in levels of β-actin mRNA molecules, which were used as an internal control, was observed.

We next examined if plasmid DNA transfection could increase the stability of MHC class I mRNA. Since actinomycin D blocks RNA synthesis, the decrease in mRNA concentrations caused by its presence should be a measure of RNA stability. Fig. 4 shows that levels of MHC class I mRNA from non-transfected cells are slightly reduced within 10 h of treatment, and so are those from plasmid-DNA transfected cells, although the relative concentrations are different, suggesting that plasmid DNA does not change the stability of MHC class I mRNA. The half-life of α-actin mRNA under the same conditions was approximately 1.5 h.

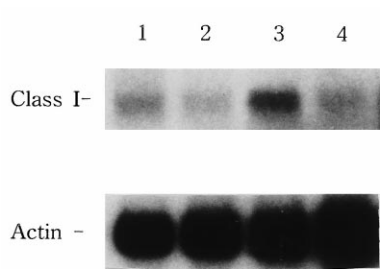

Fig. 3. Increase of MHC class I mRNA by plasmid DNA transfection. MCA-102 cells were transfected under different conditions, cultured for 48 h, and harvested for RNA. Twenty μg of cytoplasmic RNA were size-fractionated by formaldehyde/agarose gel electrophoresis, and hybridized to probes for murine MHC class I H-2K^b or α-actin. Lane 1: Medium; lane 2: liposome; lane 3: 1 μg pcDNA3.1 plus liposome; lane 4: 1 μg pcDNA3.1 pre-treated with DNase I plus liposome.

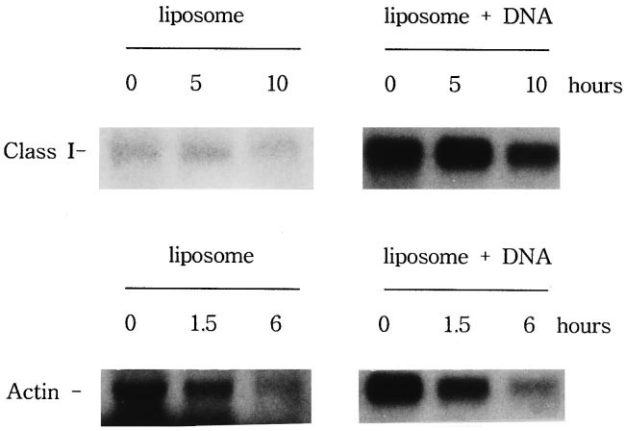

Fig. 4. Actinomycin D treatment of MCA-102 cells. 2×10^5 MCA-102 cells were transfected in the absence or presence of 10 μg pcDNA3.1. Forty-eight h after transfection, actinomycin D (5 μg/ml; Sigma) was added to the medium of the cell cultures for the indicated periods and harvested for RNA. Twenty μg of cytoplasmic RNA were size-fractionated by formaldehyde/agarose gel electrophoresis, and hybridized to probes for murine MHC class I H-2K^b or α-actin.

We next examined whether expression of genes other than the MHC class I genes is modulated by DNA. Neither surface expression of class II MHC antigens (data not shown) nor the level of β-actin RNA (Fig. 3) in MCA-102 cells was changed by DNA transfection.

4. Discussion

In the present study, we have demonstrated that the surface expression of MHC class I antigens in tumor cell lines of non-hematopoietic origin is up-regulated by plasmid DNA, only when transfection was facilitated by liposome or electroporation. However, neither the surface expression of class II MHC antigens nor levels of actin RNA are modulated, suggesting that the effects of DNA on gene expression are gene-specific. Plasmid DNA seems to exert its effects on MHC class I expression at the level of transcription.

In contrast to previous reports in which the mere presence of bacterial DNA in culture medium was able to enhance immune responses [14–16], in our study no discernable effect on MHC class I expression was induced by spontaneous transfection of plasmid DNA. The cell types previously used are of hematopoietic origin including macrophages and dendritic cells, which are able to take up extracellular antigens. In fact, internalization of bacterial DNA rather than DNA binding to a cell surface receptor was necessary for proper activation of macrophages [28]. However, the non-hematopoietic tumor cell lines used in the present study, which lack inherent

phagocytic ability, might be able to modulate expression of certain genes like MHC class I if provided with plasmid DNA intracellulary via liposome or electroporation.

In this study, we have demonstrated that the increased cell surface expression of MHC class I antigens by plasmid DNA transfection is closely associated with an increase of MHC class I mRNA. It was previously reported that in dendritic cells bacteria induced synthesis of MHC class I molecules and increased the stability of MHC class I proteins [11]. However, they did not examine transcriptional regulation of MHC class I molecules. In this study, we have shown that the half-life of MHC class I mRNA is not changed by plasmid DNA transfection. MHC class I expression is regulated mainly at the transcriptional level [6], although there are some exceptions [29,30]. Stacey et al. [28] showed that bacterial DNA induced TNF-α mRNA and increased transcription factor NF-κB DNA binding activity in macrophages. In our study, transfection of MCA-102 cells with plasmid DNA showed no effects on the mRNA level of TNF-α, IFN-α and IFN-γ genes and on the biological activity of TNF-α (data not shown). When we measured NF-κB activity under the conditions previously reported [20,31], MCA-102 cells constitutively expressed NF-κB activity which was not further modulated by DNA transfection (data not shown). At present, we do not know the molecular mechanisms of the increased expression of MHC class I mRNA by plasmid DNA transfection in non-hematopoietic tumor cells used in the present study. We speculate that plasmid DNA enhances the expression of MHC class I mRNA via activating some transcription factors other than NF-κB, which are currently under investigation.

In our study, the expression of MHC class II antigens and β-actin in MCA-102 cells was not modulated by DNA transfection, suggesting that the effect of plasmid DNA on gene expression is gene-specific. At present, we do not know how many other genes are responding to transfected plasmid DNA. If the effect of plasmid DNA is gene-specific, the question is 'Does the up-regulation of MHC class I molecules by plasmid DNA transfection in non-hematopoietic cells contribute to immunity to invading pathogens?'. Many tumor cell lines have been shown to present tumor associated antigens directly to $CD4^+$ and $CD8^+$ T cells [32,33]. Immunity against most bacteria is exerted by phagocytic cells including macrophages and neutrophils. However, many bacterial species such as *Legionella dumoffii* and *Helicobacter pylori* can grow in epithelial cells [34,35], which may act as antigen presenting cells after bacterial infection.

In the flow cytometric analysis of MHC class I antigens in the present study, almost 100% of MCA-102 and CT-26 cells treated with plasmid DNA by liposome or electroporation showed increased expression of MHC class I antigens. It was previously reported that liposome and electroporation efficiently transfected many kinds of mammalian cells at a very high frequency up to 95–100% [36,37], which may account for the findings in this study. Alternatively, some other factors, other than TNF-α, IFN-α and IFN-γ which are known to increase expression of MHC class I antigens, may be responsible for the augmentation of MHC class I expression by plasmid DNA transfection. We are currently examining the possibility.

Many types of human cancer are deficient of MHC class I molecules and can be curable by MHC class I gene transfection [2,3]. Although we do not know the molecular mechanisms of the enhancing effects of plasmid DNA on the surface expression of MHC class I genes, it may be of practical interest to use exogenous DNA in cancer therapy.

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