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Influence of E1-Deleted Recombinant Adenoviruses on B7.1 and IL-2 Expression in C1498 Cells

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ABSTRACT

Background: Knowing that adenoviral vectors could initiate innate immunity, the ability of E1-deleted recombinant adenovirus (Ad-E1Δ) in induction of B7.1 and IL-2 molecules was studied. **Methods:** The expression of green fluorescent protein in C1498 cells following transfection of these cells with adenovirus green fluorescent protein vector confirmed the ability of adenovirus vectors in infecting the cells and inducing the expression of the gene of interest. The expression of B7.1 molecule on the surface of the cells was assayed upon infection with Ad-E1Δ vector. Adenovirus-IL-2/B7.1 vector capable of inducing IL-2 and B7.1 expression in the cells was used as the positive control vector. **Results:** According to the FACS results, about 4.17% of normal cells expressed B7.1 on their surface, while this level was increased in Ad-E1Δ transduced cells up to 14.43%. These results demonstrate that Ad-E1Δ vector considerably (about 3 folds) increases the expression of B7.1 on the cells. No detectable IL-2 was secreted into the medium of non-transduced and Ad-E1Δ transduced cells. **Conclusion:** Data indicate that the infection of C1498 cells with recombinant adenoviruses stimulates expression of B7.1 on the cell surface rather than secretion of IL-2 into the medium. *Iran. Biomed. J. 11 (3): 153-160, 2007*

Keywords: B7.1, IL-2, E1-deleted, Adenovirus, C1498 cells

INTRODUCTION

Adenoviruses have been initially described in 1950s [1, 2], ever since have been characterized extensively. Nevertheless, a number of molecular mechanisms, particularly the interaction of the viral gene products with the cellular components, are obscure and the true nature of the cell response to infection is required to be further investigated. Adenoviruses are non-enveloped viruses with icosahedral symmetry, containing a linear double-stranded DNA genome encoding necessary proteins for their life cycle [3, 4]. Adenoviruses have a wide host-range and are currently divided into three genera with further subdivision into species (also termed subgenera or subgroups) A to F. Division of human serotypes, based mainly on immunological criteria, has been historically the basis of classification [5-7].

Human adenoviruses (serotypes 2 and 5) are extensively used both *in vitro* and *in vivo* and even in different human clinical trials due to their ability to infect a wide range of cells and tissues and also their potency to be produced to high titers [7-10]. Therapeutic adenoviral vectors are defective of the E1 region to provide enough space to insert therapeutic genes and to prevent virus replication in transduced cells. For their advantages, adenoviruses have been used historically as the pioneer viral vectors to treat genetic diseases such as haemophilia [11] and hypercholesterolemia [12] in animal models.

T-cell response is one of the most important mechanisms in tumour rejection [13]. A number of studies have shown that the expression of B7.1 (CD80) or B7.2 (CD86) can activate cytotoxic T cells against tumour cells [14-17] via T cells mediated mechanism depending on the tumour model [18-23]. T cell receptor interaction with MHC-antigen complex in the absence of appropriate costimulatory signals via B7 family results in onset of anergic T cells [24, 25] and evidence for

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development of tumour-specific anergy has been shown in a number of tumour models. A number of studies have revealed that the expression of B7.1 and high doses administration of IL-2 are able to prevent onset of anergy and reverse established anergy [26, 27]. This is because the combined expression of both IL-2 and B7.1 molecules enables the tumour cells to provide the co-stimulatory function normally supplied by antigen presenting cells, and to bypass T-helper cell requirement by producing IL-2. Therefore, this approach has been developed as a gene therapy strategy using viral vectors mainly adenoviral vectors to reject tumour cells.

Lance *et al.* [28] tried to transfer and express p53 in bladder tumour using recombinant adenoviral vector. Their study showed stimulation of an immune response following administration of viral vector without significant expression of p53 in the cells. Therefore, it was concluded that a part of the immune response following administration of viral vector could be addressed to the presence of vector in the body. Considering that a large number of studies have been conducted and are currently under process to use adenoviruses as viral vectors to deliver therapeutic genes into the cells in a wide range of animal models and clinical trials. Also paying attention to this point that the vector itself triggers immunological responses [29-31], encouraged us to evaluate the expression of B7.1 and IL-2 following transduction of E1 deleted type 5 adenoviruses. IL-2 and B7.1 molecules were chosen in this study because they are considered as two main immune stimulatory molecules involved in immune response initiation and more important in tumour rejection as described earlier. It should be noted that this virus is used as a backbone to construct recombinant adenoviruses encoding various genes inserted in E1 region of the virus and understanding the biology of the host immune response against replication-defective adenoviral vectors that is very important in developing these vectors towards more effective clinical vehicles.

MATERIALS AND METHODS

Cell lines. C1498 and 293 cell lines (obtained from Dept. of Haematology, King's College London) were employed in this study. The C1498 cell line is a leukemic cell line from a mouse origin and 293 (CTRL-1573) cell line is a human embryonic kidney cell transformed with E1 sequence (left end sequences) of adenovirus type 5 [32]. Both cell lines were cultured and maintained in RPMI supplemented with 5% FCS, 0.1 mM non-essential amino acids and 2 mM L-glutamine, and were incubated in a humid chamber with 5% CO_2at 37ºC.

E1-deleted recombinant adenovirus (Ad-E1Δ) vector. Ad-E1Δ (obtained from Dept. of Haematology, King's College London) is a replication-defective derivative of adenovirus type 5 Ad-E1Δ vector (which makes the backbone of the recombinant adenovirus vectors). It was used to study the induction of IL-2 secretion and B7.1 cell surface expression following transduction of the cells with adenoviral vectors. There is no cDNA insert in place of E1 in this vector. Therefore, no protein is encoded by this vector which is under the control of cytomegalovirus (CMV) promoter (Fig. 1a).

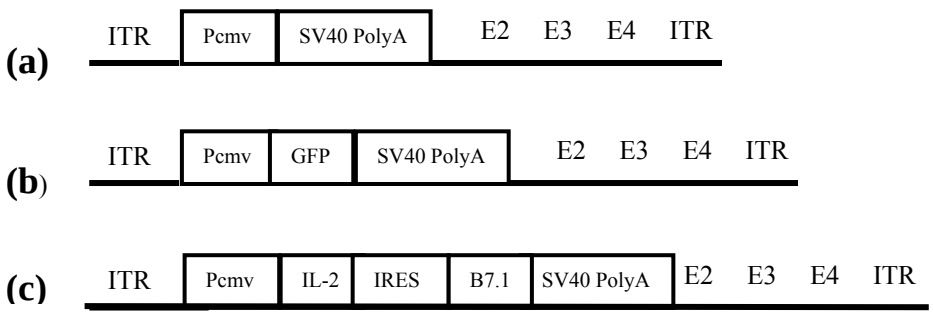

Fig. 1. Schematic representation of three recombinant adenovirus vectors. **(a)**, Replication-defective cassette of adenovirus type 5 (Ad-ΔE1) lacking E1 sequence which consisted of Ad5 inverted terminal repeat (ITR), Simian virus 40 poly adenylation region SV40 PolyA, E2/E3/E4 encoding sequences of Ad5 and ITR of Ad5. This vector is abbreviated to Ad5-ΔE1. The boxes are designated regarding of the size of their word contents not the actual length of the sequences. **(b)**, Green fluorescent protein (GFP) expressing adenovirus which is called Ad5-GFP. **(c)**, Ad5-IL-2/B7.1 vector with Ad5-ΔE1 backbone in which IL-2, encephalomyocarditis virus internal ribosome entry site and B7.1 encoding sequences are inserted in place of the GFP encoding region.

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Adenovirus green fluorescent protein (Ad-GFP) vector. AD-GFP (obtained from Dept. of Haematology, King's College London) is a replication-defective derivative of adenovirus type 5 vector lacking E1 sequence that was used as positive control vector to evaluate the permissiveness of C1498 cells to adenovirus vectors. This vector was constructed using pCA13 plasmid and encodes GFP driven by CMV promoter (Fig. 1b).

Adenovirus IL-2/internal ribosome entry site (IRES)/B7.1 (Ad-IL-2/B7.1). Ad-IL-2/B7.1 (obtained from Dept. of Haematology, King's College London) is a replication-defective derivative of adenovirus type 5 lacking E1 sequence and containing IL-2/B7.1 bicystronic fusion genes joint with encephalomyocarditis virus IRES. These fusion genes are driven by CMV promoter (Fig. 1c). This vector was used as positive control to evaluate the ability of the cells in expression of IL-2 and B7.1 molecules.

Amplification of the viral vectors. Initial propagation of the replication-competent adenovirus vectors was carried out in 293 cells upon transfection. These cells are stably transfected with E1 sequence of Ad5 and could generate progeny Ad5 viruses following transfection of replication-defective Ad-E1Δ vectors. The cells were examined daily for cytopathic effect (CPE). Then the adenovirus vectors were harvested by freeze-thawing three times and were purified using Cesium Chloride (CsCl) gradient [33], and were stored at -80ºC for future experiments. Viral vectors were propagated on 293 cells using previously prepared inoculums. The 293 cells were allowed to reach 80% confluency in 150-mm dishes and were then infected with each virus at a multiplicity of infection of 10. After absorption of the viruses at room temperature for 2 hours, adequate amounts of the culture media were added to each dish and samples were incubated at 37ºC in a humid chamber supplemented with 5% CO_2 for 3-4 days to develop viral CPE. The cells were examined daily for CPE and the adenovirus vectors were harvested by freeze-thawing three times. The viruses were purified over a CsCl gradient. Desired adenovirus band was collected and filtered fractions were then collected for each 8 droplets and their optical density (OD) was measured. The fractions with the highest OD (at the first pick) containing pure adenovirus virions without CsCl, were pooled. Then, 0.1% BSA was added to the purified adenovirus suspension and was stored at -80ºC for future experiments.

Quantification of adenovirus vectors. Virions were quantified using OD measurement and endpoint titration methods. OD of the samples were measured at 260 nm of wavelength in which every unit of OD equated to 1×10^{12} plaque forming units per millilitre. Fifty percent of tissue culture infectious dose of each virus preparation was also determined in 293 cells.

Analysis of GFP and B7.1 expression. Expression of GFP on the surface of the cells was determined by flow cytometry using a Becton Dickinson FACScan machine (USA). In this method, the green emitted colour was detected. Expression of B7.1 (CD80) on 293 cells was also determined by direct antibody labelling method. Cells were treated with a mouse anti-human-B7.1 monoclonal antibody conjugated with phytoerythrin reagent emitting red colour which allows detection of B7.1 on cell surface.

Quantification of IL-2 secretion. The expression of IL-2 driven by CMV promoter was identified by the presence of secreted IL-2 in the supernatant of the Ad-IL-2/B7.1 infected cells. An ELISA assay was employed to quantify the secreted IL-2. Genzyme hIL-2 kit containing a mouse monoclonal anti-human-IL-2 antibody was used to capture the IL-2 in the cell supernatants and a biotinylated goat anti-human-IL-2 Mab and horseradish peroxidase conjugated streptavidin were used in two steps to quantify the secreted IL-2. The assay was developed by using tetramethyl-benzidine dihydrochloride and the intensity of blue colour after adding sulphuric acid (2 M) was identified at 450 nm. Known amounts of human IL-2 were used to draw the standard curve. The assay was able to detect 15ρg/ml of IL-2.

RESULTS

C1498 cells are permissive for Ad5 vectors. To demonstrate the permissiveness of C1498 cells for Ad5 vectors, the cells were infected with Ad-ΔE1 and Ad-GFP vectors, and were examined for the expression of GFP protein. GFP was detected on the cells by FACS analysis. As illustrated in Figure 2, GFP emission was detected on 0.81% and 0.89% of the total and gated uninfected C1498 cells, respectively. The results also show that GFP emission on the ΔE1-Ad infected cells was 1.02% and 0.81% of total and the gated cells, respectively.

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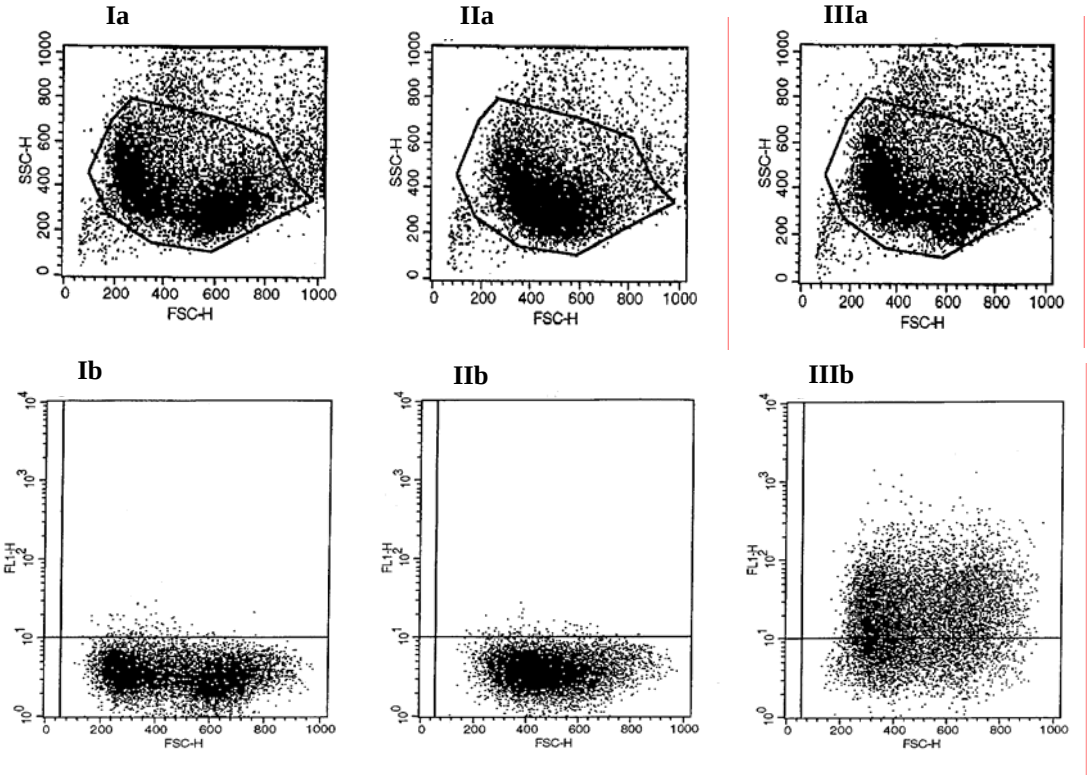

Ic				IIc				IIIc			
Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total	Quad	Events	%Gated	%Total
UL	0	0.00	0.00	UL	0	0.00	0.00	UL	0	0.00	0.00
UR	107	1.02	0.81	UR	90	0.89	0.81	UR	6818	65.78	54.09
LL	0	0.00	0.00	LL	0	0.00	0.00	LL	0	0.00	0.00
LR	10365	98.98	78.01	LR	10075	99.11	90.59	LR	3543	35.22	28.14

Fig. 2. GFP expression on uninfected (I), Ad- ΔE1(II) and Ad-GFP(III) transduced C1498 cells. The expression of GFP molecule on the surface of uninfected (I), Ad- ΔE1 (II) and Ad-GFP (III) infected cells was quantified by flowcytometery analysis. Dot plots showing the light forward scatter (FSC-H) indicating the size of the cells and side scatter (SSC-H) showing the granularity of the cells (Ia, IIa, and IIIa) were used to select proper population for further analysis. Forward scatter and intensity of the expressed fluorescent by the cells (Ib, IIb, IIIb). The percentage of the cells emitting the green fluorescent (Ic, IIc, and IIIc). GFP was detected on the 0.81% and 1.02% of the total and gated uninfected cells, respectively and on 0.81% and 0.89% of the total and gated Ad-ΔE1 transduced cells, respectively. The 54.09% and 65.78% of the total and gated Ad-ΔE1 infected cells expressed GFP, respectively.

This was while the GFP emission from the cells increased dramatically upon infection of the cells by GFP encoding adenovirus vector (Ad-GFP), where 54.09% of the total cells and 65.78% of the gated cells expressed GFP. These results clearly demonstrated that C1498 cells are permissiveness for Ad5 vectors, in terms of virus entry and viral genome replication**.**

Human B7.1 expression arises upon transduction of C1498 cells with ΔE1-Ad vector. Having confirmed the permissiveness of C1498 cells to adenoviruses according to the GFP emission difference between the Ad-GFP infected cells with uninfected and Ad-ΔE1 infected cells, the experiment was proceeded to determine whether the recombinant Ad-hIL-2/B7.1 vector could induce expression of human B7.1 on the cells. Therefore, the cells were transduced with this vector and the expression of B7.1 on the cells was analyzed. As shown in Figure 3, the expression of B7.1 molecule was detected on the surface of 4.17% and 2.44% of total and gated normal cells, respectively. In the cells infected with Ad-IL-2/B7.1 vector, the expression of B7.1 molecule was remarkably increased to 38.24% and 15.52% of the gated and

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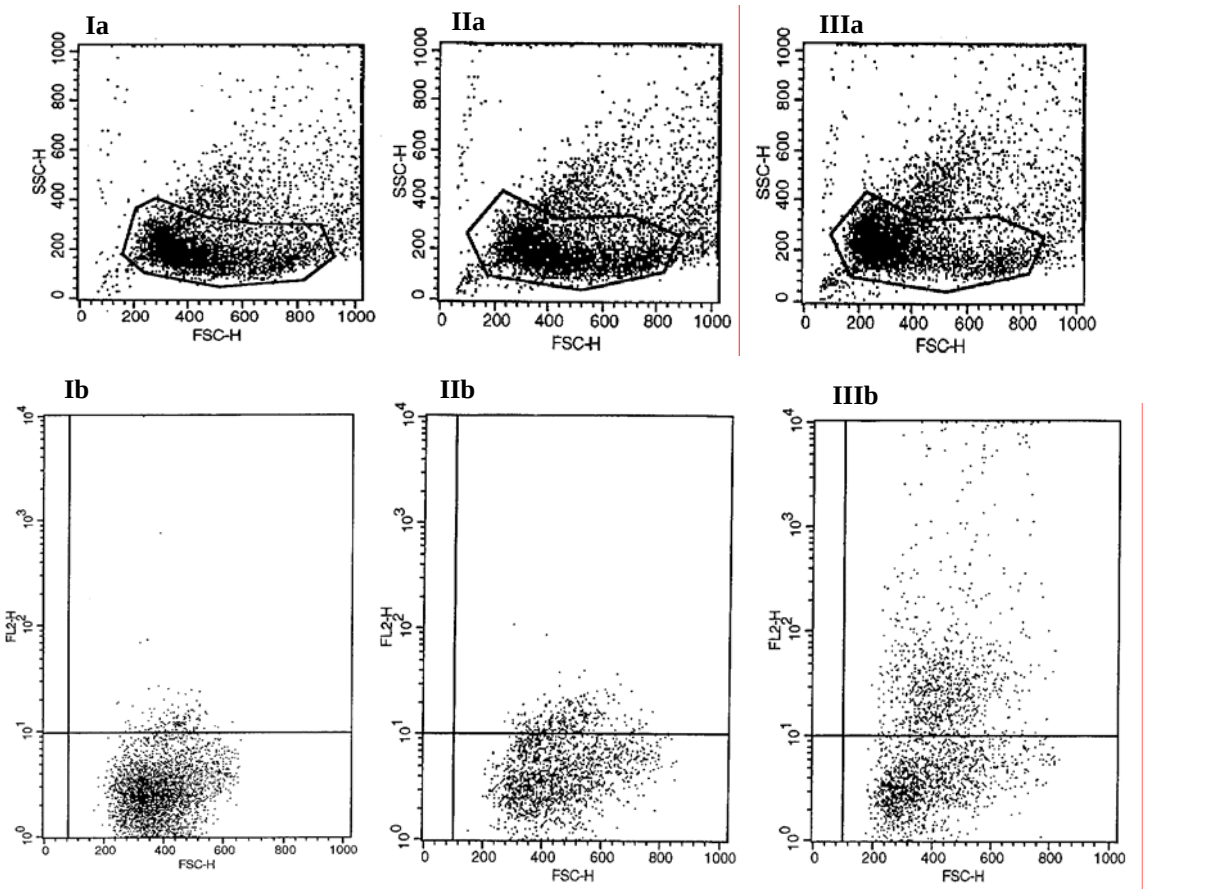

Ic			
Quad	**Events**	**%Gated**	**%Total**
UL	0	0.00	0.00
UR	142	4.17	2.44
LL	0	0.00	0.00
LR	3267	95.83	56.6

IIc			
Quad	**Events**	**%Gated**	**%Total**
UL	0	0.00	0.00
UR	285	14.43	4.60
LL	0	0.00	0.00
LR	1690	85.57	27.30

IIIc			
Quad	**Events**	**%Gated**	**%Total**
UL	0	0.00	0.00
UR	928	38.24	15.52
LL	0	0.00	0.00
LR	1499	61.76	25.07

Fig. 3. Expression of B7.1 on the surface of uninfected (I), Ad-ΔE1(II) and Ad-IL-2/B7.1(III) infected C1498 cells. Dot plot showing the light forward scatter (FSC-H) indicating the size of the cells and side scatter (SSC-H) indicating the granularity of the cells was generated for each flowcytometery assay. Enough number of events were counted and the population for each assay was selected for further analysis (panel a including Ia, IIa and IIIa). Analysis of the red fluorescent in the gated cells (panel b including Ib, IIb and IIIb) showed that 2.44% and 4.17% of the total and gated uninfected cells expressed B7.1, respectively (Ic). The expression of B7.1 was 4.6% and 14.43% of the total and gated Ad-ΔE1 cells, respectively (IIc). The 15.52% and 38.24% of the total and gated Ad-IL-2/B7.1 infected cells expressed B7.1, respectively (IIIc).

total cells, respectively. This level is about 9-fold higher than its expression on the uninfected cells. This result clearly demonstrates that C1498 cells could express B7.1 at high level and the expressed molecule is detectable with our method. The FACS analysis revealed that the expression of B7.1 molecule on the surface of the Ad-ΔE1 infected cells was 14.43% of the gated and 4.6% of the total cells, which is about 3-fold higher than its expression on the uninfected cells. This experiment clearly demonstrates that Ad-ΔE1 vector transduction results in B7.1 expression induction and increases the expression of B7.1 molecule on the cells.

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IL-2 secretion was induced by Ad-IL-2/B7.1 vector in C1498 cells. A total number of 10^6 C1498 cells transfected with Ad-IL-2/B7.1 vector were present in the assay and the supernatants of three normal and Ad-IL-2/B7.1 infected cells were assessed using ELISA technique. This experiment was carried out as an internal positive control to show the ability of C1498 cells to secrete IL-2 upon transfection and to demonstrate that this secretion is detectable by our IL-2 assay. The assay was repeated three times. No detectable hIL-2 molecules secreted into the media by uninfected cells, while the amount of hIL-2 in the supernatant of Ad-IL-2/B7.1 infected cells was 150 ρg/ml/10^6 cells/24 hours, indicating that C1498 cells are capable of expressing and secreting IL-2 into the media.

IL-2 secretion was not detected upon transduction of C1498 cells with Ad-ΔE1 vector. In order to examine the induction of IL-2 secretion following Ad-ΔE1 transduction, the cells were transduced with the vector and IL-2 secretion was quantified in triplicate using ELISA assay. According to ELISA result, no detectable hIL-2 molecules secreted into the media by Ad-ΔE1 infected cells, indicating that the level of IL-2 secretion is not increased remarkably following infection with Ad-ΔE1 vector.

DISCUSSION

In a large number of gene therapy protocols, Ad-E1Δ is used as the viral vectors. The recombinant vector itself triggers immunological responses [29-31]. Although, immune response stimulation could be considered as an advantage in some procedures [28], it is accounted as a disadvantage for viral vectors, because immune response eliminates the vector from the body and consequently shortens the viral storage time and requires repetitive vector delivery or even enables readministration the same serotype [34]. Considering that IL-2 and B7.1 molecules play key roles in immune response stimulation against viruses and tumour cells [13-17, 26, 27], in this study we investigated expression of IL-2 and B7.1 in C1498 cells following transduction with Ad-E1Δ. To achieve this goal, Ad-ΔE1 was used as the sample vector and untransduced cells were used as negative control cells. Ad-IL-2/B7.1 was used as the positive control to evaluate the ability of the cells in expression of IL-2 and B7.1 molecules. Ad-GFP vector was used as positive control vector to evaluate the permissiveness of C1498 cells to adenovirus vectors.

These results showed that adenovirus GFP could successfully transduce C1498 cells and induce the expression of GFP on the cells (Fig. 2). This indicates that adenovirus based viral vectors can attach to the cell surface receptors, be internalized by the target cell and finally insert their genome inside the nucleus. On the base of these results, the cells were infected with the prepared Ad-E1Δ vector and expressed B7.1 molecule on the surface of the cells was assayed. According to the FACS results, about 4.17% of normal cells expressed B7.1 on their surface, while this level was increased in Ad-E1Δ transduced cells up to 14.43%. These results demonstrate that Ad-E1Δ vector (about 3 folds) increases the expression of B7.1 on the cells (Fig. 3). The increase in the expression of B7.1 on the Ad-E1Δ molecule transduced cells is likely to be due to the infection. In fact, viral infection stimulates the cells to express co-stimulatory B7.1 molecule on their surface, and this makes them to be recognized by T cells.

IL-2 analysis assay was carried out using hIL-2 ELISA kit. The outcome results showed no detectable IL-2 secreted into the medium of non-transduced and Ad-E1Δ transduced cells, indicating that the infection of C1498 cells with recombinant adenoviruses stimulates expression of B7.1 on the cell surface more than the stimulation of IL-2 secretion into the medium. These results suggest that Ad-ΔE1 viral vectors have more effect on elevation of B7.1 surface co-stimulatory molecule than on the increase of IL-2 secretion. This effect may be a reflection of their role in immune response stimulation against E1-deleted adenoviral vectors. More studies with different cell lines and various cytokine expression analyses are suggested.

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