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Localization of Transfected B7-1 (CD80) DNA In Human Melanoma Cells After Particle-Mediated Gene Transfer

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Abstract

The purpose of this study was to evaluate stable DNA transfection of M-21 human melanoma cells with particle-mediated gene transfer (PMGT) with B7-1 cDNA and to identify sites of gene integration. Stable B7-1 transfectants (M-21-B7) were obtained with PMGT using a plasmid vector containing cDNA for both B7-1 and neomycin phosphotransferase, with subsequent selection with G418. The transfected cells were flow sorted by B7-1 expression into two populations, bright and dim. The bright population had 85%–90% of cells expressing B7-1; the dim population had less than 50% of cells with B7-1 expression. Chromosome analysis with fluorescence in situ hybridization (FISH) and G-banding showed that 70% of bright cells had two main integration sites, with extensive amplification of the transgene. The dim population had random signal distribution, with little or no amplification, despite G418 selection. Because B7-1 has been mapped to 3q21, FISH was performed using a chromosome 3 painting probe (WCP) together with a probe for B7-1. In transfected bright M-21 cells, amplified genes that hybridized with the B7-1 construct were localized to chromosome 3 material inserted into marker chromosomes. These data suggest that B7-1 insertion may involve homologous recombination, but maintenance of integration and amplification required selection.

1. Introduction

Various forms of gene transfer, including both viral and physical means of gene delivery, are available for the molecular modification of human melanoma cells. Viral forms of gene delivery have been extensively characterized and include viral vectors such as retrovirus, adenovirus, adeno-associated virus, and others [1], [2], [3], [4]. Many of these vectors have been shown to integrate into the chromosomes of recipient cells, thus allowing for stable transfection and long-term expression of the transgene. Integration mediated by some retroviruses, such as the murine leukemia virus, requires dividing cells [1], but DNA integration mediated by other retroviruses, such as lentivirus, can occur in nondividing cells [4]. Transgene integration mediated by retroviruses is a random event in the host genome [1], but transgene integration mediated by wild-type adeno-associated virus can occur in a defined site on human chromosome 19 [2], [3]. Although adenoviral vectors can be used to efficiently transfer genes into a variety of cell types, including cells that are not dividing, the transgene delivered by adenovirus usually remains episomal [1].

Particle-mediated gene transfer (PMGT) for the molecular modification of mammalian cells uses a hand-held particle acceleration device to deliver DNA-coated gold particles directly into recipient cells [5], [6]. Many different transgenes, including those encoding various cytokines, the HLA-A2 molecule, and the B7-1 costimulatory molecule, have been successfully delivered to a variety of mammalian cells with PMGT [5], [6], [7], [8], [9], [10]. Stable transfectants can also be obtained following delivery of the cDNA of interest in a plasmid vector containing neomycin phosphotransferase and subsequent culture in selection media containing G418 [11]; however, the fate of DNA delivered by physical means, such as PMGT, has not been clearly described [5], [6], [12]. Therefore, we undertook a study of the stability and localization of transfected cDNA following PMGT into human melanoma cells to determine the fate of the transfected cDNA following this form of gene transfer.

2. Methods

2.1. Cell line and culture conditions

The M-21 melanoma cell line was a gift from Dr. Ralph Reisfeld (Scripps Research Institute, La Jolla, CA, USA). The karyotype is pseudotetraploid (92–106 chromosomes) and extensively rearranged, with ∼18 identifiable translocations, deletions, or isochromosomes and ∼16 marker chromosomes, some of which contain regions that can be identified. Cells were cultured in RPMI-1640 supplemented with 25 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer), 25 mmol l-glutamine, 10,000 U/mL penicillin, 10,000 U/mL streptomycin, and 8% fetal calf serum (FC-RPMI). The stably transfected M-21 cells were cultured in FC-RPMI containing 1 mg/mL G418 (Mediatech, Herndon, VA, USA).

2.2. Expression vectors

Human B7-1 cDNA was obtained as previously described [11]. This expression vector contains the cytomegalovirus (CMV) promoter, the SV40 splice donor–splice acceptor region and poly-A region, and the kanamycin-resistance gene. A vector (plasmid 6535), which also contains cDNA for neomycin phosphotransferase (neo), was used to generate stable transfectants for B7-1.

2.3. Particle-mediated gene transfer

The method for PMGT used in this study was as previously reported [8], [11]. The PowderJect XR-1 particle delivery device (McCabe, 1995 patent) was used for transfection. Plasmid DNA was precipitated onto 0.95-μm gold particles (Degussa Corp., South Plainfield, NJ, USA) at a ratio of 2.5 μg plasmid DNA per mg of gold particles; 0.5 mg gold (1.25 μg DNA) was used per shot. The DNA–gold complex was accelerated through an expansion chamber using a helium pulse between 300–400 psi to accelerate the DNA–gold into target cells (1×10^6) suspended in 15 μL media. The transiently expressing cells were evaluated 24 hours following gene transfer without in vitro selection. After 12 weeks of in vitro culture under G418 selection, the stable transfectants were flow sorted. Three separate aliquots of M-21 cells were sequentially transfected with the B7-1–neo plasmid 6535 and cultured under G418 selection.

2.4. Flow cytometry and cell sorting

The M-21 cells were characterized for expression of the B7-1 molecule (CD80; Becton Dickinson, Franklin Lakes, NJ, USA) with flow cytometry as previously described [5], [11]. Analysis was limited to live cells as detected with propidium iodide exclusion. Data were acquired and analyzed using Cell Quest software (Becton Dickinson). Cells were sorted on the Becton Dickinson FACStar Plus cell sorter based on brightness of B7-1 staining.

2.5. Chromosome preparation

Metaphase spreads were obtained from transfected and nontransfected M-21 melanoma cells. The preparation of G-banded metaphase slides was performed as previously described [13]. After the location of each metaphase was noted, the slides were destained in two changes of Carnoy's fixative for 5 minutes each and used for fluorescence in situ hybridization (FISH) analysis.

2.6. FISH

Plasmid DNA containing both 1.2 kb of B7-1 and the selectable marker neo was labeled by means of nick translation using digoxigenin-11-dUTP (Boehringer Mannheim, Roche Diagnostics, Mannheim, Germany). Hybridization was accomplished in a mixture of 50% formamide–2× standard saline citrate (SSC)–10% dextran sulfate (pH 7.0), 50–150 ng labeled probe, 140 μg/mL human Cot-1 DNA, and 50 μg/mL sonicated herring sperm DNA. Hybridization, washing, and detection were performed as previously described [14], with the addition of a 30-minute incubation in 0.01 mg/mL RNase A in 2× SSC at 36°C to remove potential mRNA background signals. Chromosome 3 painting probe (WCP 3; Vysis, Downers Grove, IL, USA) was cohybridized with the B7-1 cDNA

probe by combining 1 μL WCP 3 in 7 μL WCP buffer with 50–150 ng labeled B7-1 probe. Stably transfected M-21 cells were also cohybridized with the B7-1 cDNA probe and WCP 15 or 17.

2.7. Statistical analysis

A two-tailed Student's *t*-test with unequal variance was used to compare the number of chromosome 3 containing chromosomes, the number of painted chromosome 3 containing regions that contained the labeled B7-1 probe, and the number of single or clustered signals for the B7-1 probe in the different transfected cell lines.

3. Results

3.1. Expression of the B7-1 transgene

In the first experiment, transient transfection was obtained following in vitro PMGT of M-21 cells with B7-1 cDNA, with gold particles located in the surrounding media as well as in the cytoplasm and nuclei of the target M-21 cells. As we have previously reported [8], transient B7-1 transgene expression can be detected in 8%–31% of M-21 cells following PMGT, with peak expression observed 24–48 hours following gene transfer. Transgene expression by the transfected M-21 cells declined after 48 hours in vitro and approached baseline within 1 week following PMGT (data not shown).

In the second experiment, stable transfection was obtained using PMGT with a plasmid vector containing B7-1 cDNA as well as a selectable neo marker (plasmid 6535). Culturing in G418 selection media resulted in transfected M-21 cells having ∼50%–60% surface expression of B7-1 (Fig. 1B), while B7-1 expression by nontransfected M-21 cells was essentially undetectable (Fig. 1A). Flow sorting the G418 selected M-21 cells yielded a bright population (23.5% of cells) having high expression of B7-1 (Fig. 1C) and a dim population (23.8% of cells) having moderate B7-1 expression. Cells were maintained in culture for 3 months, with the neo lines (sorted and unsorted) remaining under selection with G418.

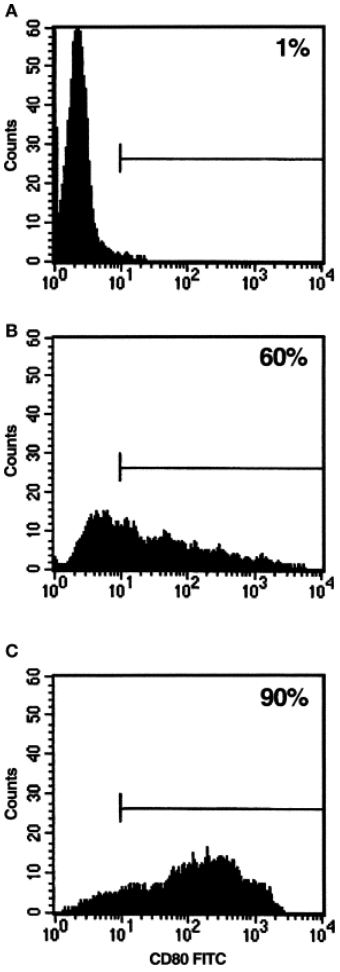

Fig. 1. Flow cytometric analysis of B7-1 (CD80) expression by M-21 cells. The B7-1 expression is shown for (A) nontransfected M-21 cells, (B) unsorted M-21-B7 stable transfectants, and (C) sorted M-21-B7 (bright) cells with high B7-1 expression. *Abbreviation*: FITC, fluorescein isothiocyanate.

3.2. Localization of transfected B7-1 DNA

At 24 hours after transient transfection, the M-21 cells showed random distribution of the transgene (Fig. 2A), with the number of signals ranging from 1 to 14 signals per cell. The stably transfected cells were flow sorted by brightness of B7-1 expression. G-banding was ambiguous with regard to identification of the integration sites; however, FISH evaluation showed that B7-1 DNA was integrated at several specific sites, with roughly 70% of the bright cells demonstrating two major integration sites (Fig. 2B). Most of the integration sites appeared to have extensive amplification of the B7-1 DNA involving marker chromosomes (Fig. 2C). A FISH analysis using chromosome 3 WCP showed that the amplified integration sites were generally limited to chromosomes containing segments derived from chromosome 3 (Fig. 2C). Because some of the markers were similar in size to chromosomes 15 and 17, WCP l5 and 17 was used and showed that no B7-1 probe signals colocalized with either the WCP 15 or 17 (data not shown).

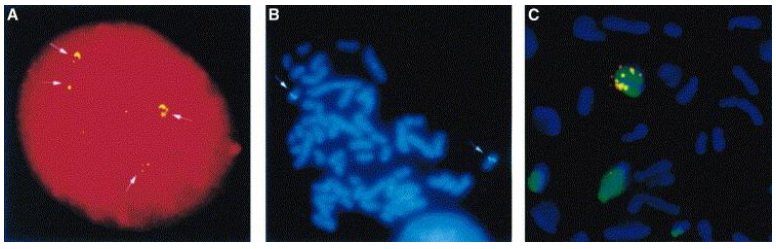

Fig. 2. Chromosomal location of the B7-1 gene in M-21 melanoma cells. (A) B7-1 DNA-coated gold particles in M-21 melanoma cell 24 hours following particle-mediated gene transfer (arrows). (B) Integration of B7-1 DNA on marker chromosomes in stable M-21-B7 cell (arrows) counterstained with 4',6-diamidino-2-phenylindole (DAPI). (C) Partial M-21-B7 metaphase showing amplification of the B7-1 transgene (red) in a marker chromosome containing chromosome 3 material (green), counterstained with DAPI (blue). Areas intensely stained with red appear as yellow dots on the green background.

The number of chromosome 3–containing chromosomes, the number of painted chromosome 3–containing regions that contained the labeled B7-1 probe, and the relationship between single or clustered signals for the B7-1 probe were then evaluated in the different transfected cell lines. Sorting and selection resulted in a significant increase in the number of chromosome 3–containing chromosomes (Fig. 3A: Unsorted M-21-B7 stable transfectants vs. Dim; $P = 0.006$). This finding occurred regardless of the level of B7-1 expression (Fig. 3A: Dim vs. Bright 1, Dim vs. Bright 2, Dim vs. Clone of 2 [where Clone of 2 is a subclone of bright population 2], and Bright 2 vs. Clone of 2; $P = 0.096$–0.545). The number of signals located in the chromosome 3–containing regions did relate to the level of B7-1 expression. The analysis for the dim cells was not significant (Unsorted vs. Dim; $P = 0.374$; data not shown); however, the analysis for the bright cells versus dim cells was significant (Dim vs. Bright 1, Dim vs. Bright 2, and Dim vs. Clone of 2; $P = 0.095$–0.001; data not shown).

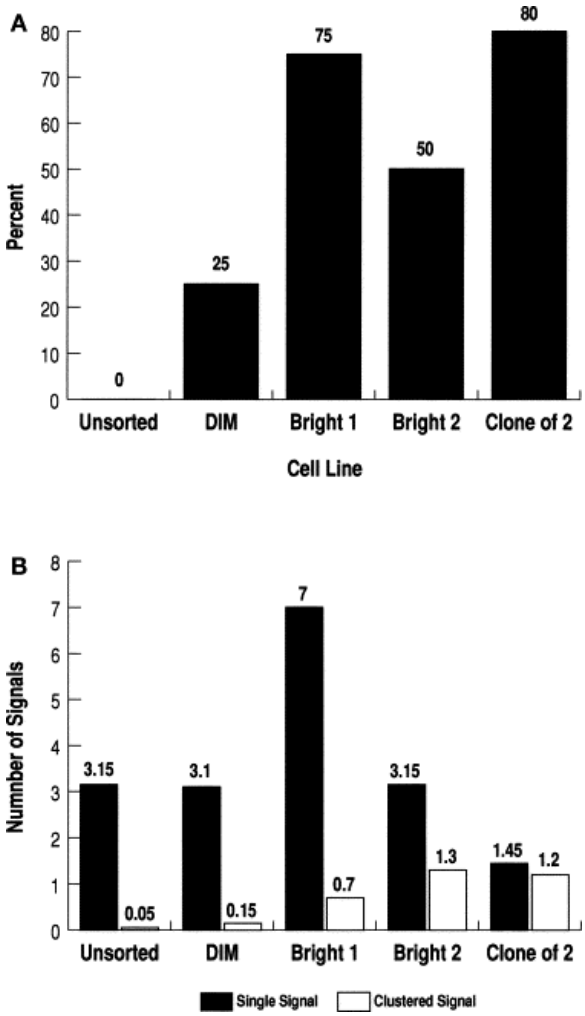

Fig. 3. Distribution of probe signals in the unsorted M-21-B7 stable transfectant cell line (Unsorted); three flow-sorted subpopulations, including M-21-B7 dim (Dim) and two bright populations (Bright 1 and Bright 2), and a subclone of Bright 2 selected for increased brightness (Clone of 2). (A) Percentage of chromosomes with B7-

1 integration into WCP 3 painted regions. (B) Number of B7-1 probe signals per cell (black lines) and number of signal clusters per cell (white bars).

The number of signals per cell was then evaluated to determine if there was a significant relationship between single or clustered signals in the different transfected cell lines. There was no significant difference in the unsorted M-21-B7 stable transfectants versus the dim cells (Fig. 3B: Unsorted vs. Dim for either single signals or clustered signals; $P = 0.887$ and 0.158, respectively). The bright cell lines, however, had a significant increase in either the number of single signals (Fig. 3B: Dim vs. Bright 1; $P = 0.001$) or clustered signals (Fig. 3B: Dim vs. Bright 2 and Dim vs. Clone of 2; $P = 0.003$ and 0.001, respectively). There was also a significant decrease in the number of single signals in the Clone of 2 compared with the sorted cell line from which it was derived (Fig. 3B; Bright 2 vs. Clone of 2; $P = 0.009$).

In summary, the number of signal clusters was much greater in the bright cells than in the dim and unsorted stably transfected cells (Fig. 3B). In contrast, there was a random distribution of the B7-1 transgene in the dim and unsorted stable transfectant cell lines without amplification, despite selection with G418 (Fig. 3B). These observations were replicated in three aliquots of M21 cells that were stably transfected to express B7-1 and sorted by brightness of B7-1 expression. We also observed that the B7-1 probe containing neo cDNA did not hybridize with chromosome 3 from normal cells, nor from nontransfected M-21 cells (data not shown).

4. Discussion

This study demonstrates that gold particles coated with a B7-1–containing plasmid vector and accelerated with a pulse of helium into target melanoma cells can be detected both in the cytoplasm and nucleus of recipient melanoma cells 24 hours following PMGT. This rapid gene transfer, as well as subsequent transgene expression within 24 hours of gene transfer, occurred without selection. We therefore conclude that plasmid DNA, present episomally within the target melanoma cells, is the source of the transient B7-1 expression in these cells, which is reproducibly present on 8%–31% of M-21 cells [8], [11].

Stable B7-1 gene expression was achieved in 50%–60% of the M-21 cells following gene transfer with a plasmid vector containing cDNA for both B7-1 and neomycin phosphotransferase, with subsequent selection in media containing G418. This contrasts to the M-21 cells with transient expression 24 hours following PMGT, in which no B7-1 DNA was detected after only 1 week in culture. The stable transfectants expressed B7-1 after being in culture with selective medium prior to this analysis.

Phenotypic analysis of the stable transfectants revealed different levels of B7-1 surface expression. The M-21-B7 cells were then flow-sorted to obtain a subpopulation of M-21 cells with high B7-1 expression. These sorted cells with high expression of B7-1 (bright) have 85%–90% of cells with detectable B7-1 surface expression. Analysis with FISH showed two major sites of chromosome integration with extensive amplification. These sites were shown to be on marker chromosomes similar in morphology to chromosomes 15 and 17. Subsequent analysis using WCP 3 revealed these marker chromosomes to contain material from chromosome 3 into which the B7-1 DNA was integrated. These findings emphasize the need for chromosome painting to verify or refine cytogenetic data obtained using traditional banding analysis. This is especially important in tumor cells with complex karyotypes [15].

The findings from the present study suggest that stable integration can occur following PMGT of B7-1 cDNA into human melanoma cells, with amplification limited to specific integration sites. Such integration appears to involve homologous recombination, because amplification of the B7-1 transgene was limited to regions with inserted chromosome 3 segments [16], [17], [18].

Although safety concerns are present with all forms of gene transfer resulting in stable transgene integration in the host genome [1], [12], this concern needs to be balanced with the benefits of stable transgene expression by the transfected cell. It appears that the DNA integration occurring after PMGT is a low-frequency event, because it required in vitro selection to identify the transfected M-21 cells with stable B7-1 integration.

In conclusion, we have shown that transgene integration into chromosomes of human melanoma cells occurs and can be detected following PMGT. In addition, the transient gene expression detected immediately following PMGT, without in vitro selection, is most likely from episomal plasmid DNA, whereas stable integration required a selectable marker. The FISH probes based on the construct containing the neo marker (plasmid 6535) readily hybridized with the inserted transgene. Amplification correlated with increased expression and was limited to chromosome 3 regions, implying homology. The M-21 line had extensive cytogenetic rearrangements that were documented prior to PMGT, including insertions of chromosome 3 material into marker chromosomes resembling 15 and 17. The presence of rearranged markers may facilitate integration and amplification, because selection for expression did not yield an increased number of normal number 3 chromosomes, whereas it did select for cells with markers containing amplified B7-1 sequences. This observation suggests that rearranged chromosome segments are more permissive to transgene insertion and amplification than are unaltered chromosomes. This has interesting implications for evolutionary mechanisms.

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