ORIGINAL RESEARCH

Potential of the immunotherapeutic effect of autologous dendritic cells by pretreating hepatocellular carcinoma with low-dose radiation

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Abstract

Purpose: To determine whether exposing hepatocellular carcinoma (HCC) to low dose radiation increases the efficacy of dendritic cell-mediated immunotherapy for HCC.

Methods: Tumour specimens collected from 20 recruited patients with HCC were cultured in primary culture (half successfully) and then exposed to low-dose radiation (0.5 Gy). Immature DCs derived from peripheral blood monocytes of patients were pulsed with autologous HCC cell lysates and matured with a cytokine cocktail. Autologous tumour lysate-pulsed DCs (TLP-DCs) were used to stimulate mixed lymphocytes, which were then tested for inhibitory effect on the growth of HCC cells. Surface markers of immunogenicity on primary HCC cells, MHC, and Fas were investigated before and after low-dose irradiation.

Results: Exposing HCC cells to low-dose (0.5 Gy) radiation enhanced the immunotherapeutic effect of TLP-DC-stimulated lymphocytes. Growth inhibition increased from 50.6±7.5% without irradiation to 74.3±4.3% with radiation. The expression of MHC class II and Fas was upregulated after irradiating HCC cells.

Conclusion: Exposing tumour cells to a low dose of radiation can enhance the immunotherapeutic effect of the autologous tumor lysate-pulsed DC vaccine.

List of Abbreviations

CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T-lymphocyte
DC-SIGN	DC-specific ICAM-grabbing non-integrin
DC	dendritic cell
HCC	hepatocellular carcinoma
imDCs	immature DCs
MLR	mixed leukocyte reaction
NLDC	normal lymphocytes lysate pulsed DCs
rhGM-CSF	recombinant human granulocyte macrophage colony stimulating factor

rhIL-4	recombinant human interleukin-4
SEM	standard error of the mean
TLPDCs	tumour lysate-pulsed DCs
UPDC	unpulsed DCs

Dendritic cells (DC) are the most potent antigen-presenting cells in the human body. They are involved in the regulation of both innate and adaptive immune responses.¹ It has been postulated that matured DCs pulsed with tumour-associated antigens may induce cytotoxic T-lymphocytes (CTLs) against tumour cells and thereby inhibit tumor growth.² Although preclinical results with tumour vaccines based on this principle are promising,^{3,4} clinical results in patients with advanced solid cancers remain disappointing with response rates less than 15%.^{5,6}

One strategy to improve the DC-mediated immunoresponse is to enhance immunogenicity by exposing the targeted tumour cells to ionizing irradiation. In addition to its direct anti-tumour effect, radiation therapy was shown to increase the presentation of antigens and upregulate the immune response.⁷ Recently, the combination of high-dose local tumour radiation (6 to 10 Gy) and adoptive DC immunotherapy has been tested in animal models of pancreatic cancer and melanoma, in sarcoma cell lines, and in patients with advanced liver cancer.^{6,8-10} While the strategy is appealing, it is not without limitation. First, when disease is advanced or metastatic, localized high dose irradiation is likely only to be palliative, since other tumours outside the radiation field may not be affected. Second, the underlying functional impairment of the host, such as liver cirrhosis, may limit the radiation tolerance of tumour-bearing organs or tissues. Conceivably, if radiation could be delivered to all tumour cells, combining ionizing irradiation with adoptive DC immunotherapy might have greater clinical impact. If proven to be feasible and efficacious, DC-mediated immunotherapy can be used not only in late-stage cancer patients but also to eradicate microscopic metastatic foci before they become clinically evident.

HCC is a common malignancy in Asia and has been getting more prevalent worldwide. The disease is diagnosed in an advanced stage in more than 50% of patients, when its prognosis is poor and treatment is ineffective.^{11,12} Therefore it is an ideal candidate for DC-mediated immunotherapy. Here, we report that low dose irradiation of HCC cells enhanced the immunotherapeutic effect of DC-mediated therapy, which may in part be attributed to the increase in MHC class II and Fas expression on HCC cells.

Materials and Methods

Patients and primary cultures

The study protocol was approved by the Institutional Review Board and Ethics Committee. From June 2005 to May 2006, 20 pre-surgical patients with HCC were enrolled in this trial. The specimens of only 10 patients (age, 46–77 yr) were cultured successfully (Table 1). Surgically removed HCC specimens were mashed, digested by DNase and collagenase, and then cultured with RPMI-1640. After two to three passages, autologous HCC tumour cells were harvested and used as the target cells. The characteristics of these autologous HCC tumour cells were compared with those of the original HCC surgical specimen by a pathologist. Hematoxylin and eosin (H&E) stained cells from the primary cultures were oval or polygonal and exhibited nuclear pleomorphism, multiple nuclei, and some mitoses. Some of them were positive for $\alpha 1$ fetoprotein. These characteristics were compatible with the original histological features of HCC (see Supplemental figure 1).

Preparation of tumour lysates

After confirming their identity as tumour cells, they were dispersed into a single-cell suspension (1×10^5 cells/mL), and then lysed by three cycles of snap freeze-thawing. Cell lysis was examined using trypan blue stain, with morphological examination under

TABLE 1. Characteristics and results of patients' lymphocyte stimulation and hepatoma inhibition.

Pt. no.	Age (yr)	Sex	Hepatitis	AFP (ng/mL)	CLIP score	MLR TLP/C (%)	Pre R/T TLP inh.	Post R/T TLP inh.
1	76	M	NBNC	2012	1	132%	ND	ND
2	47	F	B	5	0	149%	ND	ND
3	60	F	B	22306	1	196%	ND	ND
4	61	M	C	43	0	ND	ND	ND
5	66	M	B	22	1	ND	ND	ND
6	59	M	C	15	1	ND	50.0%	75.0%
7	77	F	C	545	1	ND	31.3%	77.0%
8	74	M	C	388	0	ND	76.9%	87.7%
9	49	M	B	8	1	ND	43.1%	61.5%
10	46	M	B	62980	1	ND	51.9%	70.4%
Mean	62			8832		159%	50.6%	74.3%

CLIP, Cancer of the Liver Italian Program Staging; MLR, mixed lymphocyte reaction; TLP/C(%), tumour lysate pulsed DCs / control (proliferation rate); R/T, low dose (0.5 Gy) radiation treatment; TLP inh, inhibition rate of tumour lysate-pulsed DCs; ND, not done.

light microscopy to ensure no HCC cell remained intact.

Priming HCC cells with ionizing radiation

HCC cells were harvested from primary culture and irradiated (0.5 Gy in a single fraction; dose rate, 1.0 Gy/min) using a 6 MeV linear accelerator (Clinac® 1800, Varian Associates, Inc., Palo Alto, CA, USA). Quality assurance of the radiation treatment was carried out using a parallel plate PR-60C ionization chamber (CAPINTEL, Inc., Ramsey, NJ, USA).

Generation of DCs and morphological observation

Heparinized peripheral blood was obtained from each patient for preparation of DCs. Leukocytes were isolated by Ficoll-Hypaque density centrifugation and monocytes were purified using magnetic beads conjugated to anti-CD14 and magnetic cell sorting. The harvested CD14⁺ monocytes were resuspended in RPMI-1640 (Gibco BRL, Gaithersburg, MD, USA). After 2 hr incubation at 37°C, non-adherent cells were removed and the adherent cells were collected and divided into four groups and used as indicated in different experiments. All the cells were cultured for 6 days in medium supplemented with recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF, 100 ng/mL, Schering-Plough, Kenil-

worth, NJ, USA) and recombinant human interleukin-4 (rhIL-4, 50 ng/mL, R&D Systems Inc. Minneapolis, MN, USA) every 3 days for differentiation to immature DCs. On day 6, immature DCs (imDCs) were further cultured for 24 hr without additional treatment. In the tumour-lysate pulsed cell (TLP-DC) group, immature (im)DCs harvested on day 6 were pulsed with autologous tumour lysate for 24 hr and matured with a DC maturation-triggering cytokine cocktail including IL-1 β (5 ng/mL; R&D Systems Inc.), IL-6 (15 ng/mL; R&D Systems), TNF- α (5 ng/mL; R&D Systems), and prostaglandin E2 (1 μ g/mL; Sigma, St Louis, MO, USA). In the unpulsed DC group (UP-DC), imDCs were cultured with the cytokine cocktail without tumour lysate pulsing. In some experiments, imDCs were pulsed with normal lymphocyte lysates (NL-DC) and exposed to cytokine cocktail to compare with those pulsed with tumor-lysate. For morphological assessment, DCs were stained with Liu stain and observed under a light microscope.

Analysis of surface marker expression on DCs and primary HCC tumor cells

Dual-colour immunolabelling was performed using fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs). The mouse anti-human mAbs IgG1:FITC/mouse IgG1:PE,

and appropriate isotype controls were purchased from Serotec (Oxford, UK) and used for DC characterization as follows: anti-CD14 (for IgG-FITC), anti-CD83-PE, anti-HLA-DR-PE, and anti-DC-SIGN-PE. For analysis of tumour cells, anti-MHC class I-FITC, anti-HLA-DR-PE, and anti-Fas-PE were used. Prior to fluorocytometric analysis, cells were incubated with saturating concentrations of PE-conjugated mAbs and primary CD11c mAbs followed by IgG-FITC at 4°C for 30 minutes. After washing twice with PBS, 1×10^6 cells were applied to a FACScaliber flow cytometer (BD Biosciences, San Jose, CA, USA). Data were collected and analyzed using CellQuest Software (BD Biosciences).

Autologous mixed lymphocyte reaction to DCs stimulation

To isolate mixed lymphocytes, mononuclear cells from human peripheral blood were separated by centrifugation on a density gradient (Ficoll-Hypaque, 1.077 gm/mL, Pharmacia Fine Chemicals, Piscataway, NJ, USA). After 2-hr incubation, the non-adherent cells were harvested and re-suspended in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and penicillin/streptomycin. Prior to co-culturing with mixed lymphocytes, DCs were irradiated with 30 Gy to stop proliferation and limit a potential confounding factor that might influence the primary objective (lymphocyte numbers) of this experiment. Irradiated DCs (10,000 cells) and mixed lymphocytes were co-cultured and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). After 4 days of culture, the mean fluorescence intensity of the cells was measured and analyzed by flow cytometry to determine the extent of proliferation of mixed lymphocytes.

Fluorescence microscopy

PKH 67 green fluorescence cell linker kit (Sigma-Aldrich, Inc., St Louis, MO, USA) was used to label

tumour lysate 24 hr before pulsing of immature DCs. DCs were stained with PE-labeled anti-DC-SIGN mAb for discrimination during observation under a fluorescence microscope. Microphotos were taken at 400 X magnification.

Viability of HCC cells

Mixed lymphocytes (1×10^6 cells/mL, 1 mL) were co-cultured with irradiated autologous TLP-DCs, UP-DCs, or NL-DCs (1×10^6 cells/mL, 100 μ L) for 24 hr and then subjected to co-culture with unirradiated or 0.5 Gy-irradiated autologous primary cultured HCC cells (1×10^6 cells/mL, 1 mL) for 4 days. The growth-inhibitory activity of DC-stimulated mixed lymphocytes against primary HCC cells was estimated using the trypan blue exclusion test or by the MTT assay. The tumour inhibition rate was calculated using the following formula: $(1 - [\text{live tumor cells after reaction} / \text{live tumour cells before reaction}]) \times 100 \%$.

Statistical analysis

All data are presented as mean $\pm$ SEM. Differences among groups were determined with one-way ANOVA followed by the Tukey test. The changes in surface molecule expression with and without low dose RT exposure were determined by a paired *t*-test using the statistical software package SigmaState 3.0. The level of statistical significance was set at $P < 0.05$.

Results

Morphology of DCs and uptake of tumor lysates

The monocyte-derived cells had multiple long cytoplasmic extensions characteristic for DCs. After staining with PE-DC-SIGN and culture with PKH67-labelled tumor lysates, UP-DCs only showed red fluorescence on the cell surface (Figure 1A), whereas TLP-DCs had red fluorescence on the surface as well as green fluorescence in the cytoplasm (Figure 1B).

FIGURE 1. Tumour lysate pulsed DCs take up the tumour lysates. (A) Monocyte-derived unpulsed DCs (UP-DCs) stained with PE-DC-SIGN (red). (B) Monocyte-derived DCs pulsed with tumour lysate (TLP-DCs) took up PKH67-labeled tumor lysate (green). Magnification 400 X.

This indicates that TLP-DCs had taken up the tumor lysates.

Surface marker expression of immature and mature DCs

DCs differentiated from monocytes with or without stimulation with maturation-triggering cytokine cocktail expressed low levels of CD14 (a monocyte/macrophage marker) and high levels of HLA-DR (a MHC class II molecule). The percentage of CD14-expressing imDCs, UP-DCs, and TLP-DCs was $2.2 \pm 0.5\%$, $2.8 \pm 0.8\%$, and $11.5 \pm 4.9\%$, respectively. On the other hand, the percentage of cells positive for HLA-DR was $55.8 \pm 15.0\%$, $73.6 \pm 14.1\%$, and $71.1 \pm 11.6\%$, respectively. CD83 is a well-known surface marker of mature DCs and its expression rate in imDCs was only $14.6 \pm 2.5\%$. However, it increased to $64.4 \pm 2.6\%$ ($P < 0.05$) after stimulation with maturation-triggering cytokine cocktail (UP-DCs). Although tumour cells may release immunosuppressive cytokine and might interfere with DC maturation, in our study, tumour lysate did not interfere the CD83 expression ($66.2 \pm 10.3\%$ in TLP-DCs). The comparable CD83 expression between UP-DCs and TLP-DCs indicates that pulsing with tumor lysate did not interfere with DC maturation (Figure 2, Supplemental figure 2).

Tumour lysate-pulsed DCs stimulated mixed lymphocyte proliferation

Of the four kinds of DCs, including the normal lymphocyte lysate-pulsed DCs (with degree of stimulation 1.3 times that of the control DCs, data not shown), tumour lysate-pulsed DCs stimulated mixed lymphocyte proliferation to the greatest degree (1.6 times that of the control DCs). This indicates that TLP-DCs were the most auto-stimulatory.

Cytotoxic effect of DC-mediated lymphocytes against primary HCC cells

Lymphocytes cultured with TLP-DCs inhibited the growth of autologous HCC cells to a much greater extent than lymphocytes cultured with the other types of DCs ($50.6 \pm 7.5\%$ inhibition vs. $8.1 \pm 11.7\%$ with untreated lymphocytes, $1.7 \pm 8.4\%$ with UPDCs, and $10.2 \pm 7.6\%$ with NLDCs) (Figure 3). It suggests that the autologous TLP-DCs have immunotherapeutic effects on primary cultured HCC cells through the action of lymphocytes; although greater than that of the other types of DCs, the efficacy of TLP-DCs ($50.6 \pm 7.5\%$ inhibition) was only moderate.

Enhancement of immunotherapeutic effect by low dose radiation to HCC cells

Low dose radiation (0.5 Gy) had no effect on the growth of autologous HCC cells and, as mentioned above, lymphocytes cultured with TLP-DCs inhibited growth of autologous HCC cells ($50.6 \pm 7.5\%$ growth inhibition). However, preirradiating autologous HCC cells with 0.5 Gy sensitized them to TLP-DCs-mediated cytolytic activity ($74.3 \pm 4.3\%$ growth inhibition) (Figure 3).

Induction of cell surface marker expression by low dose irradiation

The surface expression of MHC class I, MHC class II, and cell death receptor Fas on tumor with or without low dose radiation were examined. As shown in figure

FIGURE 2. Surface marker expression of various kinds of DCs. (Data are from 3 - 4 separate experiments and expressed as mean $\pm$ SEM.)

4 and supplemental figure 3, low dose radiation augmented the surface expression of MHC class II and Fas.

Discussion

We successfully used rhGM-CSF and rhIL-4 to trigger monocyte differentiation into DCs with markedly increased HLA-DR expression. GM-CSF and IL-4 are often used to induce peripheral blood mononuclear

FIGURE 4. Induction of cell surface marker expression by low dose irradiation. Data are from 6 separate experiments and expressed as mean $\pm$ SEM.

FIGURE 3. Cytotoxic effect of DC-mediated lymphocytes against primary HCC cells. The individual data are from case No. 6 - 10. TLPDCs vs. RT+TLPDCs, $p < 0.05$.

cells to differentiate into DCs. IL-4-treated DCs have high endocytic activity and induce efficiently allogeneic T-cell proliferation.¹³ These immature DCs were further matured by a cytokine cocktail on day 7, with an increase in CD83 (a marker of mature DC) expression from 12% to 62%. Although, previous reports have suggested that DCs are functionally defective in patients with cancer,^{14,15} in the current study, we successfully induced maturation of DCs using the peripheral blood mononuclear cells collected from patients with HCC. We further demonstrated that autologous tumour lysate-pulsed DCs effectively stimulate mixed lymphocytes to inhibit the growth of autologous primary HCC cells. Pretreating HCC cells with low dose radiation further enhanced the immunotherapeutic efficacy of tumor lysate-pulsed DCs.

We found TLP-DCs could engulf tumour cell fragments, as demonstrated by uptake of PKH 67-labeled tumour lysates by DCs (Figure 1B). Uptake of tumour lysate proteins, processing of the proteins, and presentation of the tumour-associated antigens by DCs, are all necessary for stimulation and activation of T lymphocytes. The exact route of antigen uptake after phagocytosis of cell fragments is unclear. Proposed mechanisms include involvement of MHC class

I and II molecules¹⁶ or uptake *via* specific receptors such as DC-SIGN¹⁷ or FcγR¹⁸.

One approach to increase tumour immunogenicity is to treat tumour cells with high-dose radiation (6-10 Gy or more) to induce tumour cell death. The resulting debris serves as the source of cancer cell antigens to be taken up by DCs that subsequently present them to the immune system.¹⁹ This is presumed to be the reason that the combination of RT and DC immunotherapy showed antitumour effect in some studies.^{8,20,21} While the strategy is appealing, there are some problems. First, the underlying functional impairment of the host may limit the radiation tolerance of tumor-bearing organs or tissues. In particular, HCC patients commonly have underlying hepatic diseases such as chronic hepatitis and liver cirrhosis. Second, when their disease is already advanced or metastatic, localized high dose irradiation is likely only to be palliative, since other tumours outside the radiation field may not be affected. If radiation can be delivered to all tumour foci, the approach of priming the tumours with ionizing irradiation followed by adoptive DC immunotherapy will have a much broader clinical impact including even eradication of microscopic metastatic cancer cells before they become clinically evident.

In our study, the low dose of radiation did not decrease the viability of HCC cells. We also failed to observe any obvious changes in HCC cell morphology related to apoptosis, necrosis, or mitotic catastrophe (data not shown). Thus, cell death might not be induced by such low dose of radiation.

The auto-stimulatory effect of T-cells was only noted in DCs pulsed with tumor lysates but not in DCs pulsed with normal lymphocyte lysates. These findings provide evidence that this approach acts through a tumour-specific mechanism. We further investigated how low-dose (0.5 Gy) radiation increased the anti-tumour effect of TLP-DCs and found increased Fas and MHC class II expression on the surface of HCC cells after 0.5 Gy. Fas is a member of the death receptor family, which is involved in the extrinsic apoptotic

pathway. Furthermore, tumour cells may be hypersensitive to low-dose radiation, an effect associated with translocation of phosphatidylserine and p53-dependent apoptosis.²² Increase in expression of Fas by ionizing radiation has been reported to correlate with increase in cellular sensitivity to apoptosis.²³ Radiation at a higher dose (10 – 18 Gy) has been reported to enhance the expression of MHC class II on myeloma and ovarian cancer cells, and this enhancement is considered to increase the immunogenicity of the tumour cells.²⁴ One report demonstrated that two radiation fractions of 4 Gy could upregulate MHC class I expression on invasive glioma GL261 cells. This up-regulation of MHC-I molecule expression might explain why whole brain radiotherapy enhances the effectiveness of immunotherapy *in vivo*.²⁵ One may speculate that surface expression of MHC class II and Fas after low dose radiation, as observed in the current study, may have contributed to the increase in immune response of tumour lysate-pulsed DCs to HCCs by increasing not only immunogenicity and consequent recruitment of effector T-cells but also susceptibility to Fas-mediated cell death, so we are on going to test these mechanism in an animal model.

Conclusions

We have demonstrated that autologous TLP-DCs maintain the ability to stimulate an autologous tumour-specific mixed lymphocyte reaction that inhibits growth of autologous HCC cells. Priming HCC cells with low-dose irradiation enhanced the immunotherapeutic effect of the TLP-DCs vaccine, which may in part be attributed to the increase in MHC class II and Fas expression on HCC cells.

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SUPPLEMENTAL FIGURE 1. A representative case (No. 2 patient) showing morphology and immunohistochemical stain of hepatoma tissue and primary cultured cells. (A – B, H&E stain for tumor tissue; C – D, H&E stain for cultured cells; E, immunohistochemical stain of AFP for cultured cells. Magnification: 250 X for A and C, 400 for B and D, and 1000 X for E.)

SUPPLEMENTAL FIGURE 2. Flowcytometric histogram for surface markers of DCs from case 2.

SUPPLEMENTAL FIGURE 3. Flowcytometric histogram for surface markers of HCC cells from case 2.