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Successful immunological treatment of gallbladder cancer in India—Case report*

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Abstract: Gallbladder cancer has a poor outcome because of its anatomy and location. Often, the diagnosis is made very late due to its silent course. Post-operated cases do respond to chemotherapy but the survival is counted in months and the quality of life is further hampered due to toxicity of drugs. Immunotherapy holds good promise in non-responding cancers treated by conventional chemotherapeutic agents. Among various therapies, dendritic cell therapy is growing at rapid pace due to its acceptable rationale. It has been utilized in treating successfully resected stage III (T2, N1, M0) gallbladder cancer in one of our patients. A 48 years old lady treated with this therapy is free of metastasis with ten doses of autologous dendritic cell vaccine constructed by utilizing resected tumor lysate antigen. She has received ten doses of therapy in 14 months of her treatment. This therapy has proven to be safe and without apparent side effects. The positive clinical response obtained supports that autologous dendritic cell-based immunotherapy is a promising therapeutic approach for refractory gallbladder cancers.

Key words: Gallbladder, Cancer, Dendritic cell, Immunotherapy
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INTRODUCTION

In cancer patients, tumor immunity is generally impaired. However, tumor immunity may occur in-vivo as circulating tumor-specific antibodies, with cytotoxic T lymphocytes (CTLs) having been identified in cancer patients (Disis *et al*., 1997; Albert *et al*., 1998) suggesting that effective anti-tumor responses may be elicited through immunotherapeutic strategies. In recent years, a number of clinical trials have proved the safety and feasibility of using human dendritic cells (DCs) to prevent tumor Ags in vivo (Schlienger *et al*., 2003) and thus DC-based vaccines represent an attractive and potential approach for cancer immunotherapy and ultimately for prevention of cancer relapse (Banchereau *et al*., 2001). DCs are generated ex vivo, charged with tumor antigens, exposed to maturation stimuli and reinfused to immunize patients (Steinman and Dhodapkar, 2001; Khan and Yaqin, 2006).

Gallbladder cancer tends to be an aggressive tumor that spreads early and leads to rapid death. The clinical pessimism surrounding this cancer is due to its late presentation, lack of effective therapy and early spread by lymphatic metastasis, hematogenous metastasis, direct invasion into the liver, high propensity to seed the peritoneal surfaces after tumor spillage and cause tumor implants in biopsy tracts, abdominal wounds, and peritoneal cavity (Bartlett *et al*., 2005). Most gallbladder cancers develop in conjunction with stones, with adenocarcinomas being the vast majority (Dienstag and Isselbacher, 2001). Classically, the 5-year survival in most large series is less than 5%, with the median survival being less than 6 months (Perpetuo *et al*., 1978). Case control studies have identified a history of biliary problems, older age, and female sex as risk factor for gallbladder cancer (Ghadirian *et al*., 1993).

Gallbladder cancer in India is one of the com-

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mon entities among GI tract cancers (Batra *et al*., 2005). The gallbladder, a pear-shaped saccular organ located under the liver in the fossa has close proximity to liver at its three planes. The gallbladder wall is much thinner than that of the intestine, and lacks a circular and transverse muscle layer. The wall has mucosa, that is, an epithelial lining and lamina propria, a smooth layer analogous to the musularis mucosae of the small intestine, perimuscular connective tissue, and serosa. In contrast to the intestine, there is no submucosa. Along the attachment to the liver, no serosa exists, and the perimuscular connective tissue is continuous with the interlobular connective tissue of the liver. Cancers of the gallbladder are staged according to their depth of penetration and extent of spread. These cancers frequently spread to the liver, which is involved in 70% of patients at the time of diagnosis. Malignant tumors of the gallbladder are insidious in their growth, often metastasizing early before diagnosis is made, more so in patients who are symptomatic and have jaundice. Tumors usually perforate the gallbladder wall, eventually causing intra-abdominal metastases, carcinomatosis, and ascites (Fleming *et al*., 1997).

In this case report, we describe a 48-year-old female patient diagnosed with metastatic carcinoma gallbladder and treated by dendritic cell therapy and is free of disease for more than one year of treatment. The lady presented in January 2005 with clinical jaundice having serum bilirubin 4.5 mg/dl (normal values <1.0 mg/dl at the time of diagnosis indicating biliary stasis due to obstruction. The cancer markers namely, serum carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP), as well as hematological parameters were within normal limits. Her ultrasonography revealed chronic cholelithiasis with cholecystitis and suspected mass at the level of common hepatic duct and proximal common bile duct causing biliary obstruction. Her computerized tomography scan showed a mass lesion in fundus and neck region of gallbladder with periportal lymphadenopathy and collapsed common bile duct (Fig.1). Over a period of a few days her bilirubin rose to 5.0 mg/dl. Poor chances of successful operation as well as poor survival without surgery were explained to her. She however gave consent for the operation, and successful excision of the gallbladder was performed at the Institute of Medical Sciences, BHU, Varanasi, India. The excised specimens were submitted for histopathology and consisted of (1) resected gallbladder with attached part of liver bed along with hepatic ducts and common bile ducts revealing mucinous adenocarcinoma invading entire thickness of its wall with perineural space embolization. The resected margins of both the hepatic ducts and common bile duct were negative for malignancy. The hepatic parenchyma showed secondary changes (nutmeg appearance and deep green color) in the form of degeneration, regeneration and cholestasis and bile duct proliferation, but free of infiltration; (2) superior pancreatic lymph node, free of disease and (3) hepatoduodenal ligament with embedded node was

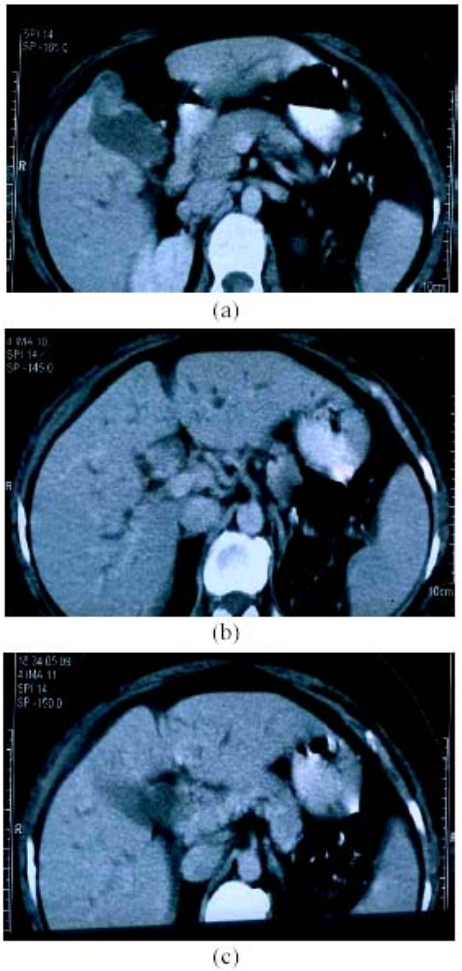


Fig.1 Pre-operative (dated February 2005). (a) Heterogenous mass involving the gallbladder fundus with direct infiltration into the adjacent liver; (b) Intrahepatic Biliary Radicular (IHBR) dilatation due to obstruction by Lymph Node at the Porta; (c) Diseased gallbladder with IHBR

processed and found to be positive for metastatic deposit. This patient was grouped in Stage III (T2, N1, M0). Histopathologically, the tumor was mucinous adenocarcinoma.

The patient soon after her operation in January 2005 consented for DC trial therapy duly approved by our Ethical Committee held on 9th July, 2004 (protocol No. 7A/9-7.04) formed as per Indian Council of Medical Research guidelines. The treatment protocol was explained to her and her family members and the first vaccine was administered in March 2005. She subsequently received six dendritic cell vaccines at one-month interval, till August 2005 and further three vaccines at one and a half-month interval till January 2006. The tenth vaccine was administered to her with an interval of three months in March 2006. Till date she has received a total of ten vaccine doses.

MATERIALS AND METHODS

Dendritic cell culture

Briefly, peripheral blood mononuclear cells (PBMCs) were extracted through Percoll (Sigma) density gradient centrifugation from 20 ml of freshly drawn blood in heparinized vials. Buffy coat was extracted and run over cell-surface treated (Nunc, Denmark) plates with added RPMI-1640 (Himedia) with complete medium, maintained at 37 °C. The complete medium consisted of RPMI-1640 supplemented with 2 mmol/L L-glutamine (HiMedia), 20 μg/ml gentamicin, 30 μg/ml amikacin and 10 μg/ml chloramphenicol. In addition to it, 10% autologous serum was used. These plates were incubated in humidified chamber, at incubation temperature for mononuclear cell adhesion. The plates were gently washed thrice with phosphate buffer saline (PBS), after incubation of one hour. Non-adherent cells were removed and adherent cells were cultured in 2.0 ml medium per well containing additionally 800 units per ml GM-CSF (R & D Systems, USA) and 500 units per ml IL-4 (R & D Systems, USA). Every other day 1.0 ml of complete medium and cytokines were added to replenish the cells.

Preparation of cell lysates from cancerous tissue

Patient's formalin preserved specimen was utilized for extracting cancerous tissue lysate antigen. Small bits were excised from the tumor tissue and washed in Phosphate Buffer Saline (PBS) five times. Gross selection of cancerous tissue was made based on macroscopic appearance. These pieces were further cut into two paired portions, one for histopathological evaluation and other for antigen extraction. Cancerous protein was extracted by subjecting 100 mg of tissue to homogenization with a syringe type, hand held homogenizer and incubated in 1 ml of RIPA buffer pH 7.6 (1 mol/L sodium di-hydrogen phosphate, 10 mmol/L disodium hydrogen phosphate, 154 mmol/L sodium chloride, 1% Triton X-100, 12 mmol/L sodium deoxycholate, 0.2% sodium azide, 0.95 mmol/L fluoride, 2 mmol/L phenylmethylsulfonyl fluoride, 50 mg/ml aprotinin (Sigma chemicals; St Louis, MO) containing 0.1% SDS at 0 °C (Ikeda *et al*., 1998). The tissue lysates were centrifuged at 15000×g for 20 min and the supernatants were collected and stored at −20 °C. Protein extracts yielded discernible protein bands ranging from 10 kD to 120 kD as identified by SDS PAGE. Western Blotting analysis was performed for detecting membrane bound proteins namely E-cadherin and cytosolic protein β-catenin and nuclear protein including proliferating cell nuclear antigen (PCNA). Lysates were further UV irradiated for 15 min and heat inactivated at 56 °C for 30 min. These lysates were mixed in isotonic saline and agitated by magnetic stirrer for 30 min. After preparation, this whole cell lysate antigenic mixture (WCLAM) was stored at –20 °C for further usage.

Antigenic exposure

After 6 d of culture, sample dendritic cells were characterized by microscopic visualization and immunofluorescence performed by confocal microscopy (Smythe *et al*., 1999). Anti-CD80 and anti-CD86 (PharMingen, San Diego, CA) antibodies were used for the study. Immature DCs were subjected to antigenic stimulation by WCLAM at ratio of 15 μl:1.0 ml of DC culture medium amounting to aggregate of 60 μl per cell plate (Khan and Yaqin, 2006). Cell culture plates were incubated further for 48 h for maturation of DCs. These cells were harvested on the 8th day of incubation. The cell medium was assessed for aerobic, anaerobic and mycoplasma contamination by routine culture and ELISA techniques. DCs were counted by trypan blue exclusion staining for cell viability and found to be 90% live.

DRII, CD83 and CD86 marker studies were performed for confirming maturation response (Fig.2). DRII assays differentiated immune modulating from tolerogenic DCs, the former being responsible for effective anti-tumor immunity.

Cell therapy—vaccination

The patient was subjected to composite DC vaccine including monocytes growth medium in 100 ml dextrose normal saline I/V drip with ondansetrone 2 mg cover. Before vaccination hypersensitivity skin test was performed on volar aspect of forearm. The second vaccine followed after four weeks of first therapy as discussed above.

RESULTS

Dendritic cell (vaccine) therapy was regularly monitored and tolerated well. No immediate or delayed adverse effects were observed. Liver function tests, kidney function tests, and analysis of hematological parameters after 3, 10 and 21 d revealed that they were in normal range. The period between any two vaccines remained uneventful. After 2 months of vaccine therapy, ultrasonography of the whole abdomen revealed normalcy in and around operation site. The post-op fluid collection in the abdomen found in earlier studies disappeared this time. Subsequent monthly dendritic cell vaccines were given and the same protocol was followed in manufacturing them. Her CT scan in March 2006 revealed no active lesion in liver but with focal fatty changes in liver parenchyma, commonly noticed after hepatic surgery (Fig.3). There was no evidence of recurrence of mass in and around the operated area. The intra hepatic biliary radicular pattern showed normalcy in caliber and composition. On completion of nine vaccines in one year, she regained normal health and her weight increased by 12 kg. Her tumor markers—AFP and CEA, remained within normal limits and complete blood chemistry including complete blood counts were in normal range. Her health has shown no uneventful process till the submission of this report.

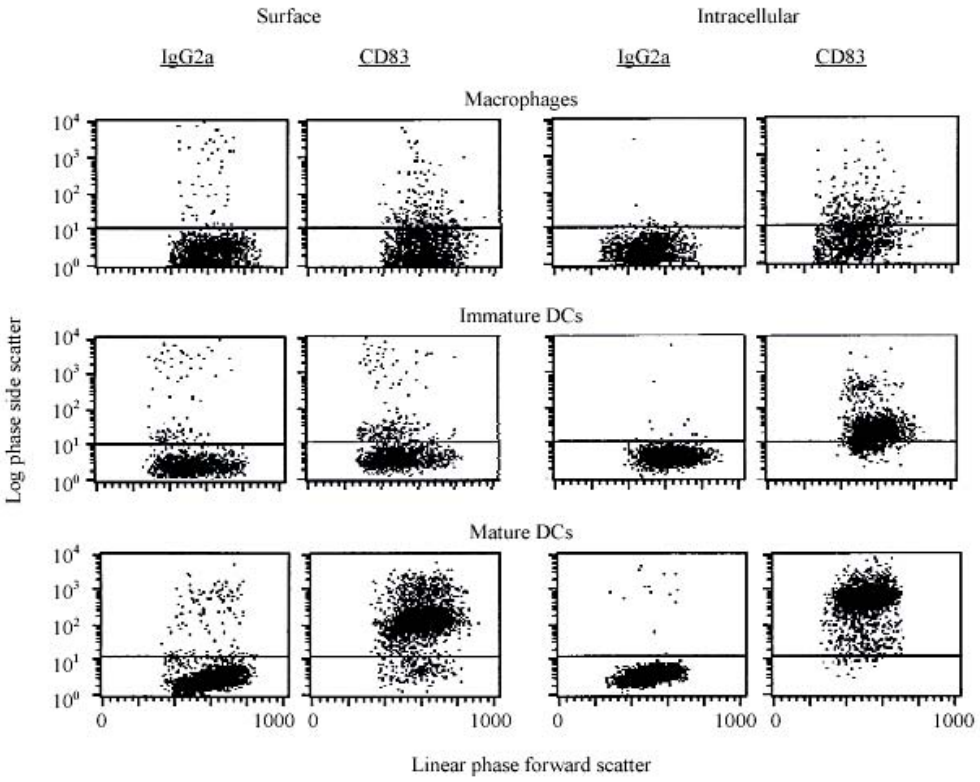
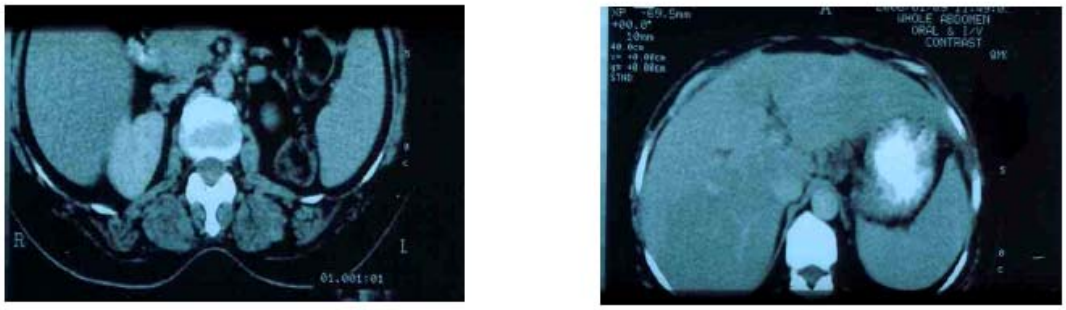


Fig.2 Extracellular CD83 expression distinguishing immature DCs from mature DCs and macrophages depicted by florescence activated cell sorter scan



(a) (b)

Fig.3 Post-operative (dated March 2006). (a) Cholecystectomy with localized hepatic resection; (b) Post-vaccine therapy. No evidence of recurrence of mass. The IHBR are normal in caliber. Note is made of focal fatty changes of liver

CONCLUSION

Immunotherapy, especially dendritic cell therapy is expected to give good results in stage IV cancer. Most of the trials are conducted during this stage of disease where no therapy can be a guarantee of cure. Moreover, with enormous tumor load at this stage of disease and with chemotherapeutic interventions utilized to the fullest possible extent leading to immune suppression, dendritic cell therapy trial becomes a failure. Most of the trials including Phase III trial in hormone refractory prostatic adenocarcinoma, completed in 2005 depicted 4.5 months of improvement as compared to placebo (Srivastava, 2006). Multicentric phase III trial in patients with stage IV melanoma also failed to give satisfactory response. The cytotoxic T lymphocyte response generated after vaccine therapy is not an absolute indication of the halt in disease progression. Development of effective immune therapy will require a more comprehensive and real-time immune monitoring in various compartments and patient specific modulation of immune responses. Peptide antigens associated with Major Histocompatibility Complex (MHC) class I or class II molecules are the molecular targets for T-cell recognition of cancer. To characterize the host-tumor relationship and to optimize cancer vaccines, clinical studies using defined peptide antigens offer special opportunities to advance the field and find important place in effective immune therapeutics (Slingluff *et al*., 2006). The authors' recent experience in most of the DC vaccines indicates a positive response when (1) autologous cancerous antigen is used for maturation of DC and (2) minimal tumor load at the time of initiating therapy. Further, loading dendritic cells with self antigens having unique cancer epitopes in glioblastoma muliforme (Khan and Yaqin, 2006) and in numerous other cases (unpublished data) showing improved outcome compared to generalized cancer antigens (non-autologous) indicate unique molecular structural differences in cancers even of the same lineage. Advances in genome sequencing technologies can be of help to know the entire genomic sequence of the cancerous tissue and then to identify from this sequence a small number of relevant mutated, unique antigenic epitopes, which can then be synthesized and used to immunize directly or utilized for construction of DCs. Another concern will always be the expression of relevant epitopes as per our plan because expression is the prerogative of DCs and their unique expression is in their own control.

It is unlikely that significant improvements in the survival of patients with biliary tract cancers will be seen with currently available chemotherapeutic agents, so that new approaches are therefore needed. Gallbladder cancer is an aggressive disease with a dismal prognosis. Immunotherapy in the form of dendritic cell therapy given to this 48-year-old lady with gallbladder cancer has proved to be safe and effective. Before initiating this therapy the patient the likely benefits she may have with conventional chemotherapy and its limitations were explained to her. She was also educated on the rationale behind DC therapy and its probable outcome. The diagnosis of gallbladder cancer should not be approached with a fatalistic attitude, as appropriate workup, extended

resection and newer approaches including dendritic cell therapy can extend quality of life with chances of cure.

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