TABLE II – ISOLATED ANTIGENS BY SEREX WITH SERA FROM PATIENTS WITH PANCREATIC CANCER

Clone name	Number of isolated clone	Length of isolated clone (kbp)	Identity	Accession Number	Chromosomal locarization
f1	4	3.2	hPMS1	BF210947	Ch.2
f2	2	2.2	hMSH2	AU131598	Ch.2
f3	2	2.5	SOX13	AI014340	Ch.1
f4	2	1.4	HRY	AL552433	Ch.3
f5	2	5	KIAA0580	AU140056	Ch.4
f6	1	2.1	Translocase of inner mitochondrial membrane (TIM) 44	AU121643	Ch.19
f7	1	1.5	Galectin 9	BF347161	Ch.17
f8	1	2.5	CTCL tumor antigen se2-2	AF176816	Ch.12
f9	1	1.3	Mitochondrial ribosomal protein (MRP) L12	BG104344	Ch.17
f10	1	1.2	HS1 binding protein (HAX1)	BF212858	Ch.1
f11	1	1.6	ZNF 207	BE617467	Ch.17
s1	5	1.5	hnRNP methyltransferase (HMT)1	AU131177	Ch.19
s2	2	2.3	IMAGE: 3480396 3	BF059745	Ch.22
s3	1	2	Kinectin 1	BE816123	Ch.14
s4	1	2.3	Chromosome22 clone RP-43L2	AI814302	Ch.22
s5	1	3.2	Hypothetical protein DKFZp761 D1823	AI089375	Ch.9
s6	1	2.3	RUNX2	BF059745	Ch.6
s7	1	2.5	p53	BF342477	Ch.17

TABLE III – FREQUENCY OF SERUM IgG Ab SPECIFIC FOR THE SEREX IDENTIFIED ANTIGENS

	Healty individuals (n = 34)	Pancreatic cancer (n = 37)	Renal cell cancer (n = 8)	Esophageal cancer (n = 10)	Melanoma (n = 10)	Prostate cancer (n = 10)	Bladder cancer (n = 10)	Colon cancer		Endometrial cancer (n = 6)	Pancreatitis (n = 7)
								MSI (+) (n = 10)	MSI (−) (n = 7)		
hMSH2	0	5	1	0	0	1	0	0	0	1	0
hPMS1	0	3	1	0	0	1	0	0	0	0	0
HRY	0	3	0	1	0	1	0	0	0	0	0
SOX13	0	2	0	0	0	0	0	0	0	1	0
MRPL12	0	2	0	0	1	1	0	0	0	0	0
HMT1	0	2	0	0	0	2	0	0	0	0	0
HAX1	0	1	0	1	0	0	0	0	0	0	0
ZNF207	0	1	0	0	0	1	0	0	0	0	0
RP-43L2	0	1	0	0	1	0	0	0	0	1	0
KTN1	4	8	0	0	0	5	2	0	2	1	3
IMAGE:3480396 3′	4	5	1	1	0	1	0	2	0	0	0
KIAA0580	2	4	0	2	1	1	0	1	0	1	0
RUNX2	4	4	3	1	0	2	0	1	0	0	0
TIM44	1	2	0	0	0	1	1	1	0	1	0
p53	1	2	0	2	0	0	0	0	0	0	0

for 5 min. The products were separated by 10% SDS-PAGE, sensitized in 0.5 M sodium salicylate solution for 10 min and dried with a gel dryer. The radioisotope was detected in BAS 2500 or 5000 (Fujifilm).

Preparation of recombinant hMSH2 protein and Western blot analysis

The *hMSH2* cDNA was amplified by PCR with primers containing *Bam*H1(5′) and *Not*1(3′) sites (5′-GGATCCATGGCGGTGCAGCCGAAGGA-3′ and 5′-GCGGCCGCTCACGTAGTAACTTTTATTC-3′) from the lambda phage isolated by SEREX. The PCR products were digested with *Bam*H1 and *Not*1 (TAKARA), and ligated into the expression plasmid, pET16b. The recombinant protein was expressed in *E.coli AD494(DE3)pLys S* (Novagen) with IPTG induction, and purified by Ni^{2+} affinity chromatography, Hi Trap Chelating (Amersham Biosciences).

Western blot analysis was performed as previously described.[9] Briefly, 2.5 μg of recombinant hMSH2 protein was electrophoresed on 8.5% polyacrylamide-SDS gel and transferred onto nitrocellulose membrane. The membrane was blocked by 5% nonfat milk and incubated with sera at 1:100 dilution or mouse anti-His antibody (Amersham Biosciences) at 1:3,000 dilution and then reacted with alkalinephosphatase-conjugated goat anti-human IgG antibodies or goat anti-mouse IgG antibodies at 1:3,000 dilution. The Ab reacted bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tertazorium.

Statistical analysis

Fisher's exact test was used to evaluate correlation between complication of cancer and serum anti-hPMS1 or hMSH2 antibody in DM/PM patients.

Results

Isolation of tumor antigens by cDNA expression cloning with IgG Ab in sera from 8 patients with pancreatic ductal adenocarcinoma

A total of 1.3×10^6 clones of the cDNA library made from 5 pancreatic ductal adenocarcinoma cell lines, PK1, PK8, PK9, PK45P and PK59, were screened with 2 mixtures of sera from 4 patients (total 8 patients) with pancreatic ductal adenocarcinoma. A total of 30 cDNA clones representing 18 genes (f1–f11 with the first serum mixture and s1–s7 with the second serum mixture) was isolated (Table I). These represented 14 previously characterized and 4 uncharacterized genes. Some antigens were isolated more than 2 times. Five arginine methyltransferase *HMT1*, 4 DNA mismatch repair enzyme *hPMS1* and 2 DNA mismatch repair enzyme *hMSH2* were isolated (Table II). These isolated antigens include molecules possibly related to formation of malignant phenotypes, including

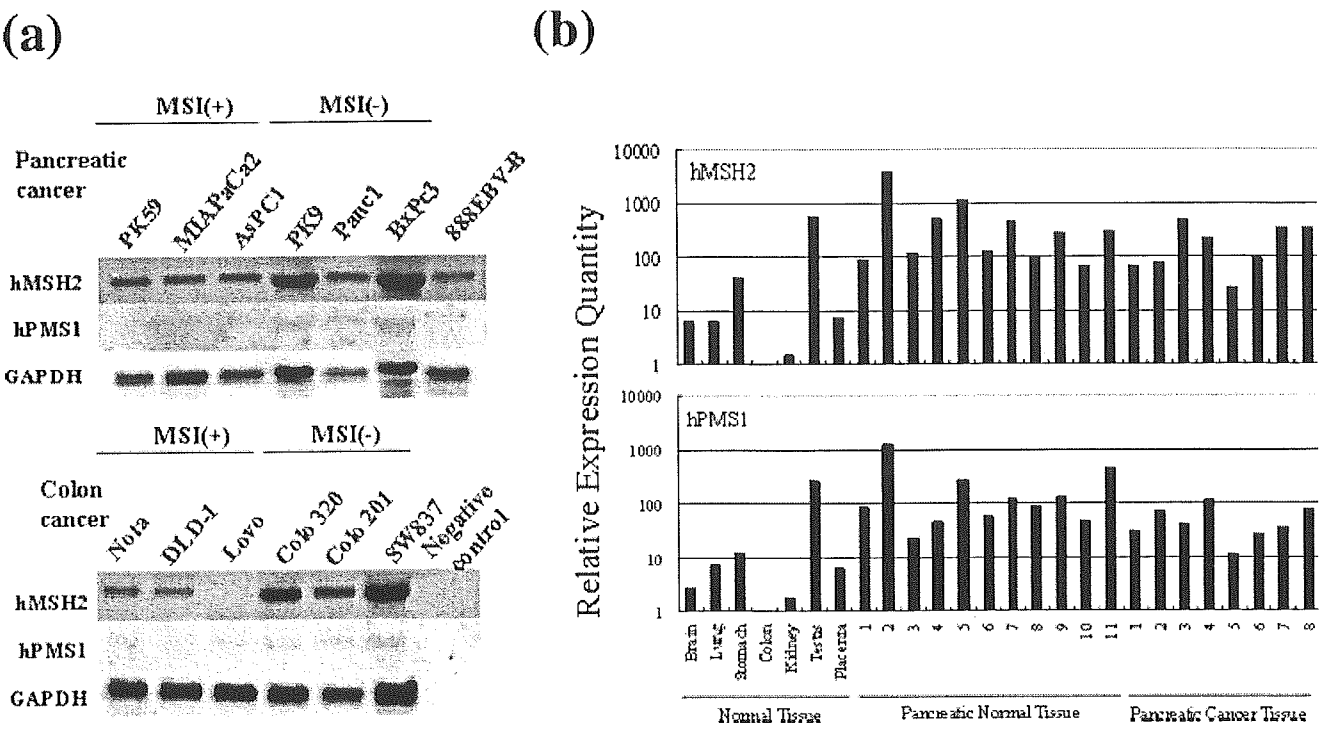

FIGURE 1 – mRNA expression of hMSH2 and hPMS1. (*a*) High expression of hMSH2 and hPMS1 in MSI negative cancer cell lines. Northern blot analysis was performed for hMSH2 and hPMS1 in MSI positive and negative cancer cell lines. Both hMSH2 and hPMS1 showed lower expression in MSI positive pancreatic ductal adenocarcinoma cell lines (PK59, MIAPaCa2 and AsPC1) and colon cancer cell lines (Nota and DLD1) than MSI negative pancreatic ductal adenocarcinoma cell lines (PK9, Panc1 and BxPC3) and colon cancer cell lines (Colo320, Colo201 and SW837). Colon cancer cell line LoVo with homozygous hMSH2 gene deletion only expressed hPMS1. (*b*) The expression of hMSH2 and hPMS1 in various normal tissues and pancreatic cancer tissues. The mRNAs for hPMS1 and hMSH2 were quantitatively measured in various normal tissues, including brain, kidney, lung, colon, stomach, testis, placenta and 11 normal pancreas tissues, and 8 pancreatic cancer tissues using TaqMan real-time RT-PCR. GAPDH was used as an internal control. The mRNAs in various samples were estimated by the 2[-Delta Delta C(T)] relative quantification method. A ratio of the mRNA of various samples to that of colon was shown.

transcription factor *SOX13*, oncogene *p53*, methyltransferase *HMT1* and DNA mismatch repair enzymes *hMSH2* and *hPMS1*.

Screening of IgG Ab reacting to the SEREX identified antigens for evaluation of their immunogenicity in sera from patients with various cancers and healthy individuals

These 18 antigens were screened for evaluation of their immunogenicity using sera from patients with various cancers and healthy individuals. To identify target antigens involved in antitumor immune responses, we selected antigens for which immune responses was raised preferentially in cancer patients. By screening each isolated antigen with serum IgG Ab from 34 healthy individuals, we first selected 15 antigens which reacted with less than 5 healthy donor sera and further evaluated their immunogenicity with sera from patients with various cancers, including 37 pancreatic ductal adenocarcinomas, 8 renal cell cancers, 10 esophageal cancers, 10 melanomas, 10 prostate cancers, 10 bladder cancers, 17 colorectal cancers (10 with MSI+ and 7 with MSI- cancer) and 6 endometrial cancers. Sera from 7 patients with acute pancreatitis were also screened to exclude possible immune response to normal pancreas (Table III).

IgG Ab specific for hMSH2, hPMS1, HRY (hairy Drosophila-homolog), SOX13 (sex determining region Y-box 13), MRPL12 (mitochondrial ribosomal protein L12), HMT1, HAX1 (HS1 binding protein), ZNF207 (zinc finger protein 207) and RP-43L2 were detected in sera from the patients with pancreatic ductal adenocarcinoma and other cancers but not detected in any sera from 34 healthy individuals or 7 patients with pancreatitis. Among these 9 antigens reacted with only sera from cancer patients, serum IgG Abs specific for hMSH2 or hPMS1 were detected in 5 of 37 (13.5%) or 3 of 37 (8.1%) patients with pancreatic ductal adenocarcinoma, respectively, and in 3 of 71 or 2 of 71 patients with other cancers, respectively.

Expression of the SEREX identified antigens in various normal tissues and cancers evaluated by RT-PCR and Northern blot analysis

Tissue specific expression of these 9 antigens for which IgG Ab were detected in sera from patients with various cancers but not in sera from healthy individuals were first evaluated by RT-PCR analysis on various normal tissues, primary cultured normal cells, cancer cell lines and pancreatic ductal adenocarcinoma tissues, and these antigens were found to express ubiquitously in various normal tissues and cancers (data not shown). However, by Northern blot analysis, *hMSH2* and *hPMS1* were found to express higher in most cancer cell lines tested, including PK9 pancreatic cancer, 526mel melanoma, KIS lung cancer, TE8 esophageal cancer, KU7 bladder cancer, MDA231 breast cancer and RCC6 renal cancer cell lines, than in various normal tissues, suggesting over-expression of *hMSH2* and *hPMS1* in various cancer cells (data not shown). Interestingly, when compared expression of these genes between MSI positive and MSI negative cancer cell lines in pancreatic ductal adenocarcinoma and colon cancer, *hMSH2* and *hPMS1* appeared to express higher in the MSI negative cancer cell lines than in MSI positive cancer cell lines, although the expression of *hPMS1* was lower than *hMSH2* (Fig. 1*a*). The colon cancer cell line LoVo with homozygous deletion of *hMSH2* expressed only *hPMS1*. Although these results may suggest possible involvement of these DNA mismatch repair enzymes in MSI status, and possible higher immune response to these enzymes in patients with MSI negative cancers, we could not detect the specific serum Ab in any colon cancer

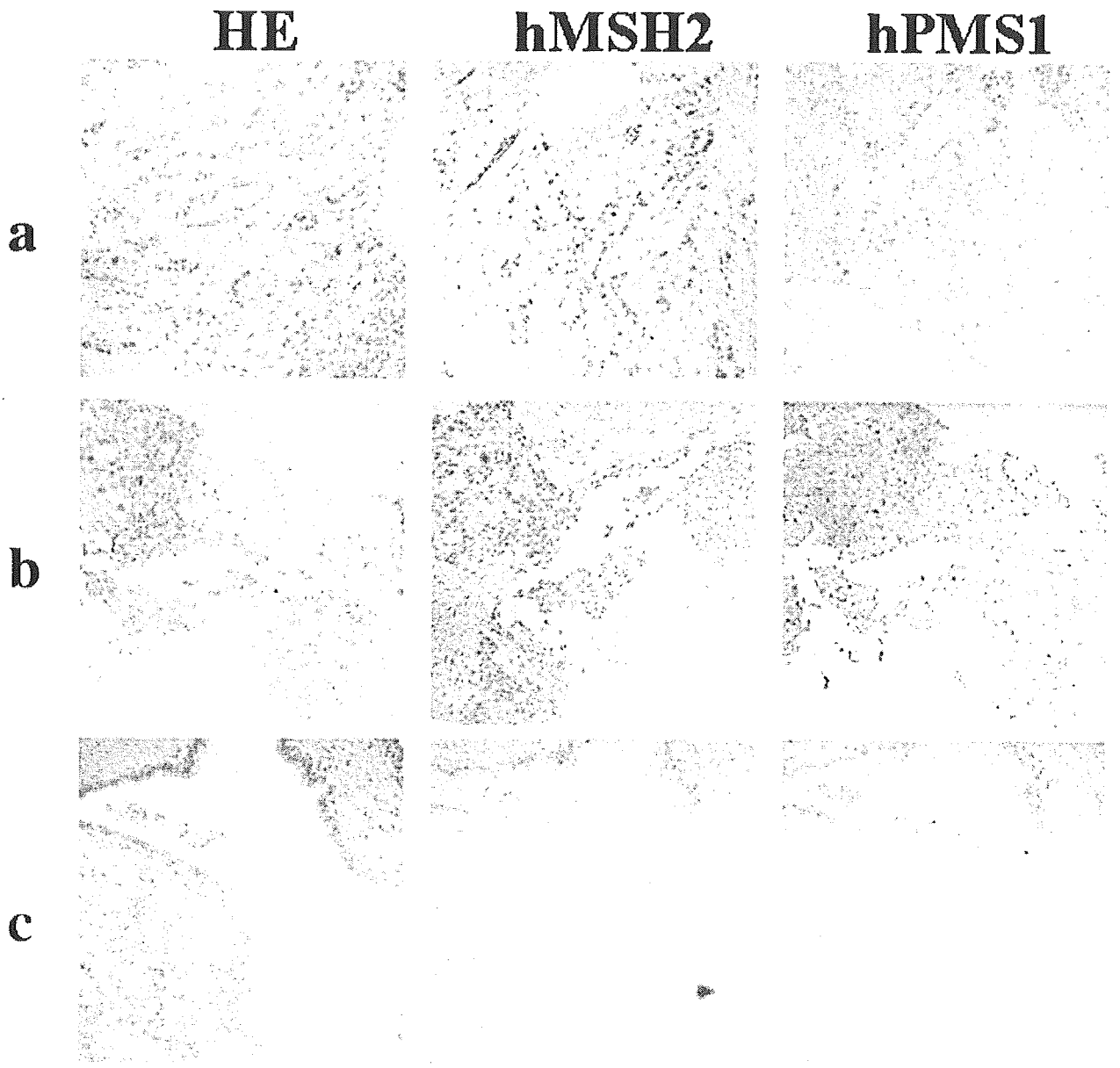

FIGURE 2 – Overexpression of the hMSH2 and hPMS1 proteins in pancreatic cancer. Immunohistochemical study was performed for hMSH2 and hPMS1 on pancreatic ductal adenocarcinoma and normal pancreas tissues. One representative pancreatic ductal adenocarcinoma (*a*) obtained from a patient with positive serum IgG Ab for both hMSH2 and hPMS1, and 1 representative pancreatic ductal adenocarcinoma (*b*) from a patient without the specific Ab was shown. One representative normal pancreas tissue (*c*) from a patient without the specific Ab was shown. The nucleus and cytoplasm of the pancreatic ductal adenocarcinoma cells were strongly stained for hMSH2 and hPMS1. No significant difference of the intensity was found between tumor cells from patients with or without serum anti-hMSH2 and hPMS1 Ab. Epithelial cells of the normal pancreatic ducts were weakly stained.

patients tested, and MSI status was unknown in the pancreatic cancer patients whose serum Ab were evaluated for their reactivity.

We then evaluated the expression of *hMSH2* and *hPMS1* in 8 pancreas cancer tissues and various normal tissues including 11 pancreas tissues using quantitative real time PCR. Among normal tissues, relatively high expression was observed in testis and pancreas. Although pancreatic cancer tissues expressed these 2 genes at relatively high amounts, no difference was observed between pancreatic cancer and normal pancreas (Fig. 1*b*). We also evaluated expression of these 2 genes using publicly available gene data bases. In the Unigene database, *hMSH2* and *hPMS1* were found in cDNA libraries from various normal tissues, including brain, lung, heart, stomach, colon, liver, kidney, testis, skin, muscle, blood, bone marrow and so on, but expression is low (227 *hMSH2* sequences and 243 *hPMS1* sequences of total 5×10^6 registered sequences). In the SAGE databases, the expression of *hMSH2* and *hPMS1* was also low in various normal tissues including pancreas. No significant difference in the expression of these 2 genes was observed between normal pancreas and pancreatic cancer, although upregulation of both genes was found in breast cancer. These results indicated that mRNA for *hMSH2* and *hPMS1* may not be overexpressed in pancreatic cancer.

Over-expression of hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma by immunohistochemical analysis

Since mRNAs evaluated by real time PCR analysis were obtained from bulk pancreatic cancer tissues that contained lots of noncancerous stromal cells, we then performed immunohistochemical study to

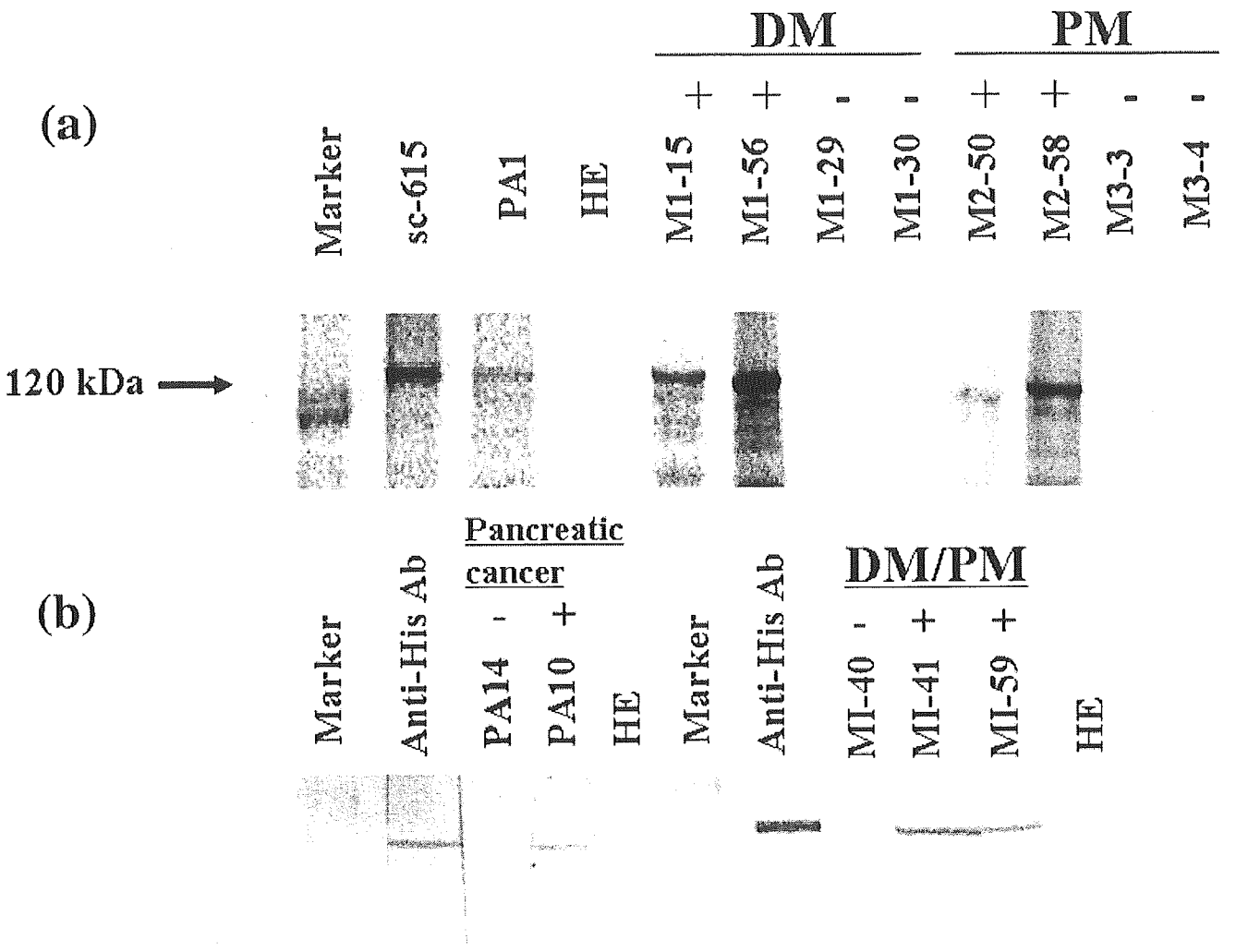

FIGURE 3 – Presence of anti-hMSH2 or hPMS1 antibody in sera from patients with DM/PM. (*a*) Anti-hPMS1 Ab was detected by immunoprecipitation in some of the patients with pancreatic cancer or DM/PM. The hPMS1 protein produced by *in vitro* transcription and translation was immunoprecipitated with anti-hPMS1 rabbit polyclonal Ab sc-615, and sera from a healthy individual (HE), a pancreatic cancer patient (PA1) and DM/PM patients (M1-15, M1-29, M1-30, M1-56, M2-50, M2-58, M3-3 and M3-4). This figure shows 1 of the representative results. The DM patient M1-15 had breast cancer. +, positive bands, −, negative bands. (*b*) Anti-hMSH2 Ab was detected by Western blot analysis in some of the patients with pancreatic cancer or DM/PM. The Western blot analysis was performed with the bacterial recombinant hMSH2 protein, anti-His tag Ab and sera from a healthy individual (HE), patients with pancreatic cancer (PA10 and PA14) and DM/PM (M1-40, M1-41 and M1-59). This figure shows 2 representative experiments. +, positive bands, −, negative bands.

TABLE IV – NO CORRELATION BETWEEN SERUM ANTI-HPMS1 AND HMSH2 IgG AB AND CANCER COMPLICATION IN DM/PM PATI

	Myositis antibody		Cancer +	Cancer −
hPMS1	PM	+	0	5
		−	0	63
	DM	+	1	2
		−	2	46
hMSH2	PM	+	0	3
		−	0	59
	DM	+	0	2
		−	2	36

evaluate the hMSH2 and hPMS1 protein in cancer cells at a single cell level. Five pancreatic ductal adenocarcinoma specimens obtained from patients who had anti-hMSH2 serum Ab, 3 specimens from patients with anti-hPMS1 serum Ab and 6 specimens from patients without either Ab were stained with anti-hMSH2 [illegible] monoclonal Ab or [illegible] anti-hMSH2 and [illegible] normal pancreatic duct cells, normal pancreas acinar [illegible] cells and stromal cells, the intensity of the nuclear and cytoplasmic staining was stronger in pancreatic ductal adenocarcinoma cells, indicating over-expression of the hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma cells [Fig. 2, 1 representative example of pancreatic cancer tissue (*a*) from a patient who had serum IgG Ab for both hMSH2 and hPMS1, and 1 representative example of pancreatic cancer tissue from a patient without the specific serum Ab (*b*) and normal pancreas tissue (*c*)]. No different intensity of the staining of pancreatic ductal adenocarcinoma was observed between the specimens from patients with or without the anti-hMSH2 and anti- hPMS1 Abs.

Detection of anti-hPMS1 and anti-hMSH2 Ab in sera from patients with dermatomyositis and polymyositis

Anti-hPMS1 Ab was previously reported as a specific autoantibody detected in approximately 7.5% (4/53) of patients with

dermatomyositis and polymyositis (DM/PM), and it was not detected among patients with systemic lupus erythematodes or sclerderma.[12] Therefore, we evaluated anti-hPMS1 and anti-hMSH2 Ab in sera from DM/PM patients. More than 100 sera from the DM/PM patients were screened for anti-hPMS1 serum Ab by a convenient rapid assay using immunoprecipitation with the S-35 methionine-labeled hPMS1 protein produced by *in vitro* transcription and translation (Fig. 3*a*). Anti-hMSH2 serum antibody was screened using Western blot analysis with the bacterial recombinant hMSH2 protein (Fig. 3*b*) because the hMSH2 protein was not well produced by *in vitro* translation. Anti-hPMS1 antibody was detected in sera from 8 of 119 (6.7%) DM/PM patients with similar frequency to the previous report.[12] Similarly, anti-hMSH2 Ab was detected in sera from 5 of 102 (4.9%) DM/PM patients. The anti-hMSH2 Ab was not detected in sera from patients with other collagen diseases, including 10 rheumatoid arthritis, 10 systemic lupus erythematodes, 10 sclerosis or patients with infectious diseases, including 10 tuberculosis and 2 brain abscesses (data not shown), indicating that anti-hMSH2 Ab may also be a specific autoantibody for DM/PM patients. Since PM/DM patients, particularly DM patients, are known to have complications of various cancers including pancreatic cancer, we have examined possible correlation between cancer complication and serum Ab specific for hPMS1/hMSH2. Although 1 DM patient with positive anti-hPMS1 serum Ab complicated with breast cancer, no significant correlation was found between cancer complication and anti-hPMS1 or anti-hMSH2 serum Ab in our analysis with more than 100 DM/PM patients (Table IV). We also examined other myositis specific autoantibodies, including Ab against aminoacyl tRNA synthetases and signal recognition particle (SRP) in all cancer patients with positive serum anti-hPMS1 Ab, but none of them were positive in the cancer patients (data not shown). Therefore, anti-hPMS1 and anti- hMSH2 Ab appeared to be raised in the DM/PM patients independent of cancer development. These results suggest that serum Ab specific for hMSH2 and hPMS1 may be useful for diagnosis of pancreatic cancer and DM/PM.

Discussion

Since pancreatic cancer is one of the problematic cancers for early diagnosis and treatment with conventional therapeutics, new diagnostic and therapeutic methods need to be developed. Clinical trials of immunotherapies attempted on pancreatic cancers demonstrated some anti-tumor effects. Intradermal immunization with the mutated K-ras peptide with GM-CSF resulted in the induction of memory CD4+ T cell response and prolonged survival in the T cell responders compared to the nonresponders.[13,14] Vaccination with GM-CSF transduced allogeneic pancreatic cancer cell lines with adjuvant radiation and chemotherapy following surgical excision demonstrated possible benefit in disease free [illegible] immunotherapy may be one of the additional therapeutic [illegible] ities for patients with pancreatic cancer. However, the mechanism for immunological tumor regression has not been well evaluated and improvement of the immunotherapy has been difficult partly because only a limited number of tumor antigens have so far been identified for pancreatic cancer.[16,17] Furthermore, additional tumor markers are required for better diagnosis of pancreatic cancer. Thus, identification of additional antigens is important for development of immunotherapy and diagnostic methods for patients with pancreatic cancer.

In our study, we have isolated tumor antigens by SEREX using the cDNA library made from 5 pancreatic ductal adenocarcinoma cell lines and 8 allogeneic sera from patients with pancreatic ductal adenocarcinoma. These identified antigens included interesting molecules possibly related to cancer phenotypes, such as transcription factor *SOX13*, oncogene *p53*, methyltransferase *HMT1* and DNA mismatch repair enzyme *hMSH2* and *hPMS1*. *SOX13* was recognized by sera from patients with 2 of 37 pancreatic cancers and 1 of 6 endometrial cancers but not recognized by any sera from 34 healthy individuals. *SOX13* is a member of the *SOX* D group that also includes *SOX5* and *SOX6*. We have previously reported that SOX5 and SOX6 were specifically over-expressed in primary brain tumors, and the HMG box regions in SOX 5 and SOX6 were frequently recognized by sera from brain tumor patients.[8] Thus, SOX13 may be expressed in some of the pancreatic cancers and recognized by IgG Ab. Alternatively, antibody raised against SOX5 or SOX6 might cross-react SOX13 because the HMG box has high homology among these *SOX* D group protein.[18] p53 is previously reported to be recognized by T cells from patients with pancreatic cancer,[19] and frequently recognized by serum IgG Ab in patients with various cancers including colon cancers.[20–22] However, the particular p53 clone isolated in our study was recognized by sera from patients with a limited cancer including pancreatic cancer and was not recognized by sera from colon cancer patients. Downregulation of *HMT1* which may upregulate antiproliferating effects of IFNs through interaction with the intracytoplasmic domain of the Type I interferon (IFN) receptor was reported in breast cancer.[23,24] HMT1 was recognized by sera from patients with 2 of 37 pancreatic cancers, 2 of 10 prostate cancers but not recognized by any sera from 34 healthy individuals. Since DNA mismatch repair enzyme hMSH2 and hPMS1 were recognized by sera from 5 of 37 and 3 of 37 patients with pancreatic cancers, respectively, but not recognized by any sera from 34 healthy individuals, they were further investigated for their expression and immunogenicity in various cancers.

Although mRNAs for *hMSH2* and *hPMS1* were ubiquitously detected by RT-PCR analysis, the immunohistochemical study revealed over-expression of the hMSH2 and hPMS1 proteins in pancreatic ductal adenocarcinoma compared to normal pancreatic tissues. Over-expression of hMSH2 in ovarian cancer cells compared to normal ovarian tissues was previously reported by immunohistochemical analysis.[25] Although the expression of hMSH2 in rapidly proliferating normal cells such as intestinal epithelial cells was reported,[26] the mechanism for over-expression in ovarian cancer was not simply explained by higher proliferative ability of cancer cells because no correlation was observed between expression of hMSH2 and Ki-67 antigen. Over-expressed antigens such as galectin9[27] and HER2[28] were previously reported to induce immune response in cancer patients and frequently detected as tumor antigens by SEREX. A heat shock protein, hsp105 over-expressed in various cancers, including pancreatic cancer and colon cancers, was previously identified by SEREX with serum from a pancreatic cancer patient.[29] Thus, over-expression of proteins in tumor cells might induce immune responses to hMSH2 and hPMS1 in patients with pancreatic cancer.

Some of the pancreatic cancers are known to have microsatellite [illegible] due to [illegible] [illegible] Genetic alterations of *hMSH2* have [illegible] Patients with MSI positive pancreatic cancer was reported to have better prognosis after treatment.[30] In our study, higher expression of hMSH2 and hPMS1 in the MSI negative cancers than the MSI positive cancers was observed in the pancreas and colon cancer cell lines. High proliferative ability of MSI negative cancer with high hMSH2 and hPMS1 expression may be associated with poor prognosis of MSI negative cancers. To investigate relationship among MSI status, hMSH2/hPMS1 expression and their specific Ab, further study on fresh cancer samples along with MSI status is necessary.

Although both $CD4^+$ and $CD8^+$ T cell responses to hMSH2 and hPMS1 remain to be investigated, these antigens may be useful as at least $CD4^+$ helper T cell antigens for immunotherapy, particularly in patients with positive serum Ab. The recognition by IgG Ab suggests that the same antigen activated $CD4^+$ helper T cells (Th) in the patients, meaning that theses antigens are

immunogenic in cancer patients. In addition, many SEREX defined antigens were shown to induce CD8+ cytotoxic T cells (CTL).[32–35] Positive correlation was observed between positive serum IgG antibody and induction of CD8+ CTL against a cancer-testis antigen NY-ESO-I.[32,36] In pancreatic cancer, SEREX defined antigen, coactosin-like protein (CLP), was reported to induce HLA-A2 restricted tumor reactive CTL from PBMC.[37] In the case of over-expressed antigens in tumor cells with relatively lower expression in normal cells, specificity of effecter T cells to cancer cells is defined by density of MHC/peptide complex on tumor cell surface. Thus, relatively low level expression of proteins in normal cells dose not exclude the use of T cells against these over-expressed antigens as previously reported in several tumor antigens including SART-1.[38]

In addition to be target antigens for immunotherapy, anti-hMSH2 and hPMS1 Ab may be useful for diagnosis of pancreatic cancer, since we have previously observed disappearance of serum antibody specific for the SEREX defined antigens in the patients who had good prognosis after treatment.[7,9] Anti-p53 antibody was reported to be utilized even for early diagnosis of esophageal cancers.[39] In our study, we also examined anti-hPMS1 and anti-hMSH2 Ab in sera from DM/PM patients, since anit-hPMS1 antibody was previously reported to be an autoantibody specifically detected in 7.5% of PM/DM patients.[12] The anti-hPMS1 Ab was detected 8 of 119 (6.7%) DM/PM patients in a similar frequency as previously reported. In addition, we found that anti-hMSH2 Ab was also detected in 5 of 102 (4.9%) DM/PM patients at a similar rate but not detected in other collagen diseases or infectious diseases, suggesting that anit-hMSH2 Ab may also be an autoantibody specific for DM/PM and useful for diagnosis of DM/PM patients. Antibodies to other DNA mismatch repair enzymes, including hMSH3, hMSH6, hPMS2 and hMLH1, remain to be investigated. The preferential induction of these Ab in patients with DM/PM and pancreatic cancer indicated that simple tissue destruction is not the reason for these Ab responses.

DM was known to be strongly associated with various cancers, although complication rate was about 10%, and PM was moderately associated with increase of cancer.[40–42] The risks of pancreatic cancer, lung carcinoma, ovarian cancer, gastric cancer, colorectal cancer and non-Hodgkin lymphoma were increased in DM patients and risks of non-Hodgkin lymphoma, lung cancer and bladder cancer were increased in PM patients.[43] Some autoantibodies are known to correlate with specific clinicopathological features in DM/PM patients, including anti-Jo-1 antibody for multiple arthritis/interstitial pneumonia and anti-SRP antibody for treatment-resistant severe myositis. Therefore, we examined whether positive anti-hPMS1/hMSH2 Ab had any correlation with cancer complication in the DM/PM patients. Although 1 patient complicated with breast cancer had serum anti-hPMS1 antibody, no significant correlation was observed among more than 100 patients, suggesting that these antibodies may be raised independently of cancer development with yet unknown mechanism. Alternatively, numbers of patients evaluated in our study are still too small to draw any conclusions, we thus would like to follow up the patients with positive serum autoantibody.

In summary, using SEREX, we identified 2 DNA mismatch repair enzymes, hMSH2 and hPMS1, which are over-expressed in pancreatic cancer cells, and whose antibodies were detected in patients with various cancers, particularly with pancreatic ductal adenocarcinoma, indicating that hMSH2 and hPMS1 are immunogenic antigens in various cancer patients and possibly useful as T cell antigens for immunotherapy. In addition, their antibodies may be useful for diagnosis of patients with both pancreatic cancer and DM/PM patients.

Acknowledgements

We thank M. Mukai for kindly providing some cancer tissues for immunohistochemistry. We also appreciate the expert technical assistance of K. Dan.

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Original Paper

European Surgical Research

Eur Surg Res 2005;37:228–234
DOI: 10.1159/000087868

Received: December 6, 2004
Accepted after revision: May 25, 2005

Irradiated Pancreatic Cancer Cells Undergo both Apoptosis and Necrosis, and Could Be Phagocytized by Dendritic Cells

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Key Words
Dendritic cells · Immunotherapy · Pancreatic cancer · Apoptosis · Irradiation

Abstract
The interaction of immature dendritic cells (DC) with irradiated pancreatic cancer cells was examined. Flow cytometric analysis using annexin V and propidium iodide revealed that ionizing radiation (25–35 Gy X-ray) induced both apoptosis and necrosis in pancreatic cancer cell lines. After irradiation, PK-1 and Panc-1 cells were likely to undergo necrosis, whereas MIAPaCa-2 cells underwent apoptosis. When DiO-stained immature DCs were co-incubated with DiI-stained irradiated MIAPaCa-2, it was observed under fluorescent microscopy that DCs phagocytized dead tumor cells as early as 4 h after co-incubation. The DCs' phagocytosis of irradiated tumor cells was also confirmed by flow cytometry. These results suggest that irradiated pancreatic cancer cells, which undergo both apoptosis and necrosis, could be a good source of tumor-associated antigens for cross-presentation by DCs.

Introduction

Dendritic cells (DCs) are bone marrow-derived leukocytes whose properties for antigen presentation and the initiation of T-cell-dependent immune responses are more potent than those of other antigen-presenting cells such as macrophages or B cells [1]. Immature DCs reside in peripheral tissues for efficient antigen capture. After antigen uptake, DCs migrate to T-cell-enriched areas of lymphoid tissue for presenting captured antigens to T cells. During migration, DCs become mature with higher expression levels of MHC class I and II, as well as co-stimulatory molecules including CD86, CD80 and CD40 [2]. Because of DCs' importance in the immune system, investigators have focused on their role in the context of antitumor immunity. It has been reported that DCs can infiltrate into tumor tissues, as demonstrated by mouse tumor models and clinical studies [3–6]. These are referred to as tumor-infiltrating dendritic cells, mimicking tumor-infiltrating lymphocytes [7]. Such tumor-infiltrating dendritic cells might enter the tumor tissue for the purpose of obtaining tumor-associated antigens (TAAs) from the tumor cells themselves.

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The mechanism by which DCs take TAAs in and present them to other immune cells is not yet well understood. However, several means of priming DCs to elicit an antitumor immune response have been examined [3]. One of the modalities to prime DCs is using irradiation. Ionizing radiation is able to induce both apoptosis and necrosis in tumor cells, and such dead cells are then captured by DCs to elicit a tumor-specific immune response [8–10].

In the present study, we chose human pancreatic cancer cells for our experiments. Pancreatic cancer, especially ductal carcinoma, is one of the most difficult cancers to treat, and so-called multidisciplinary therapy may be necessary, though unfortunately not usually effective [11]. We examined whether irradiation could kill pancreatic cancer cells, and whether DCs could phagocytize such irradiated tumor cells. This study provides indirect evidence that DCs might present TAA by utilizing phagocytized pancreatic cancer cells per se, which could lead to the rationale of irradiation-combined DC immunotherapy against pancreatic cancer.

Materials and Methods

Tumor Cell Lines

We used three human pancreatic ductal adenocarcinoma cell lines: PK-1, PANC-1, and MIAPaCa-2. PK-1 was established from resected specimens in our institute [12]. PANC-1 was a gift from Dr. M. Kobari (Sendai Open Hospital, Sendai, Japan), and MIAPaCa-2 was provided by Dr. F. Kanai (Tokyo University, Tokyo, Japan). PK-1 and PANC-1 were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, N.Y., USA) supplemented with heat-inactivated 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. For MIAPaCa-2 maintenance, Dulbecco's modified Eagle's medium (Life Technologies) was used instead of RPMI 1640. For the following experiments, all tumor cells were used in a logarithmic proliferation state.

Annexin V Assay

Apoptosis/necrosis detection assay was performed by flow cytometry using annexin V and propidium iodide (PI) [13]. Tumor cells were put in 6-well plates (Falcon™, Becton Dickinson, Franklin Lakes, N.J., USA) at the concentration of 5×10^5/ml, and were irradiated using X-ray irradiator HF-320 (Shimadzu, Kyoto, Japan) at the dose of either 25, 30, or 35 Gy. They were harvested at different time points and stained using TACS™ Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, Md., USA) according to the manufacturer's instructions. Data of the stained cells were immediately acquired by FACSCalibur (Becton Dickinson) and analyzed by CellQuest (Version 3.1F) software (Becton Dickinson).

DC Propagation

Human DCs were propagated from peripheral blood monocytes (PBM) using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (both cytokines were provided by Schering-Plough Corp., Kenilworth, N.J., USA) according to methods described elsewhere [14, 15]. Briefly, 50–80 ml peripheral blood samples were obtained from healthy donors. Buffy coats, obtained by gradient centrifugation with Ficoll-Paque plus (Amersham Biosciences, Piscataway, N.J., USA), were collected and put in a 25-cm^2 flask (Falcon™) with 10 ml of culture medium, AIM-V (Gibco Invitrogen Corp., Carlsbad, Calif., USA). After 1-hour incubation, non-adherent cells were removed, while adherent cells (PBM) were cultured with 10 ml of fresh AIM-V supplemented with 1,000 U/ml each of GM-CSF and IL-4. We exchanged one-half volume of the culture medium every other day. After 6 days' incubation, non-adherent cells were harvested, resuspended, and used as DCs in the present study. In some experiments, PBM derived from pancreatic cancer patients with informed consent were used for DC propagation.

Flow Cytometry Staining

The following fluorochrome-conjugated monoclonal antibodies (mAb) were used for flow cytometry staining to assess the phenotype of propagated DCs: FITC-anti-HLA-DR, PE-anti-CD86, FITC-anti-CD83, and PE-anti-CD14. In addition, purified anti-CD1a mAb was used with FITC-anti-mouse IgG1 mAb secondarily. To detect contaminated lymphocytes in the harvested samples, PE-anti-CD3, FITC-anti-CD4, FITC-anti-CD8, and PE-anti-CD19 mAbs were used. In each staining, isotype-matched IgG mAbs were used as negative controls. All of these mAbs were purchased from Pharmingen (San Diego, Calif., USA).

Cellular Labeling

DCs and/or tumor cells in the culture were labeled with fluorescent membrane-bound molecules, SP-$DiIC_{18}$ [3] (DiI) or SP-$DiOC_{18}$ [3] (DiO) (Molecular Probes, Eugene, Oreg., USA) according to the manufacturer's instructions. Labeled cells were extensively washed with PBS and resuspended in fresh culture media before the experiments.

Fluorescent Microscopy

We used an inverted microscope system, Eclipse TE 300 (Nikon, Tokyo, Japan), combined with HB-10103 AF (Nikon), for fluorescent microscopic examination. Photographic data were digitally acquired and analyzed using Fujix Photograb 300Z version 2.01 software (Fuji Photo Film, Tokyo, Japan).

Results

Irradiation Could Induce both Apoptosis and Necrosis in Pancreatic Cancer Cells

We, at first, examined whether irradiation could induce cell death in pancreatic cancer cells. To detect apoptosis or necrosis, we employed a flow cytometry protocol using annexin V and PI [13]. When PK-1 was irradiated at 30 Gy, both apoptosis (annexin V-positive, PI-nega-

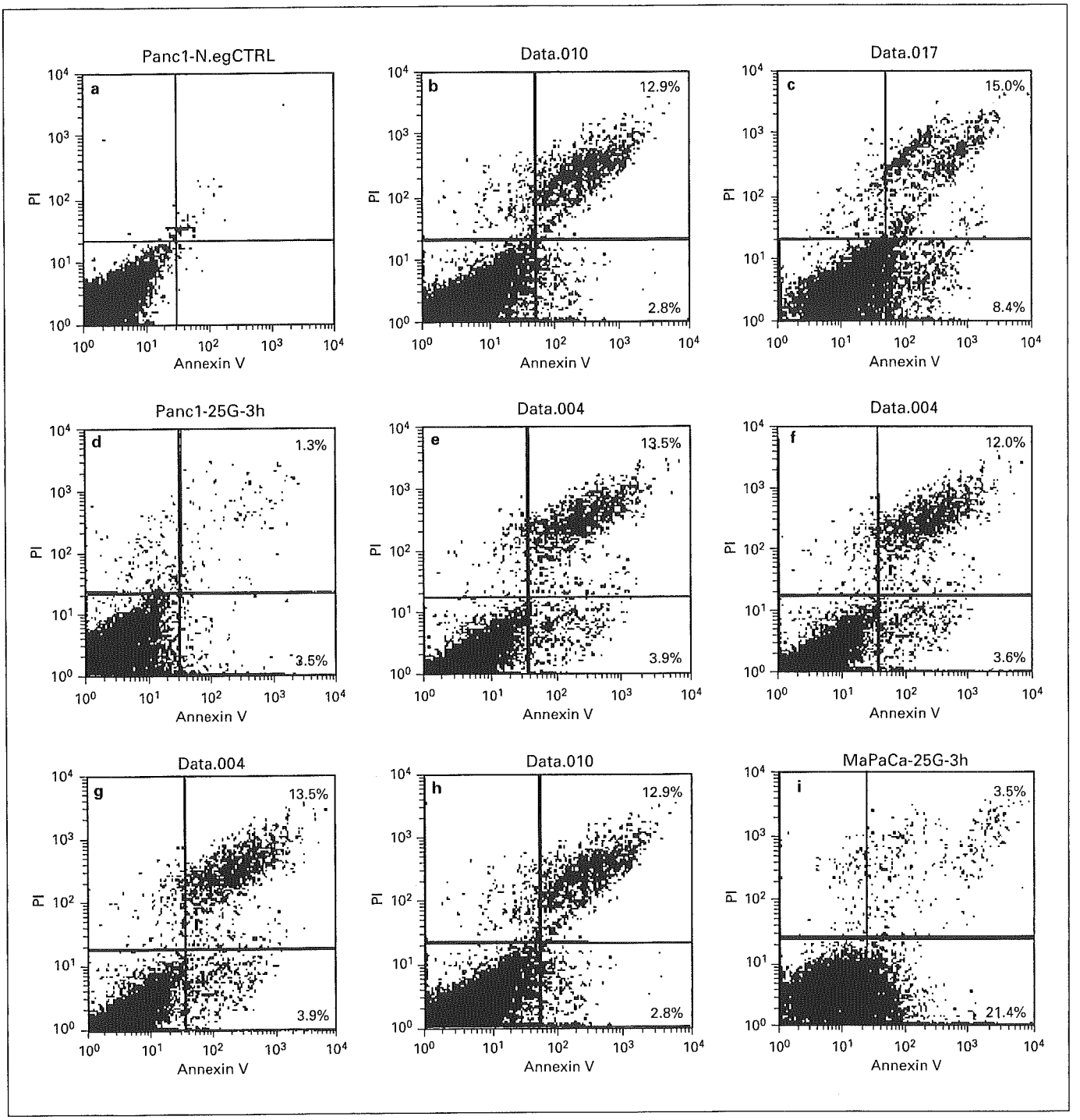

Fig. 1. Apoptosis/necrosis detection assay by flow cytometry using annexin V and PI. **a** Representative negative control. **b**, **c** Time course assay 3 h (**b**) and 6 h (**c**) after irradiation. **d–f** Radiation dose escalation (3 h after irradiation) at 25 Gy (**d**), 30 Gy (**e**), and 35 Gy (**f**). **g–i** Different cell lines (3 h after irradiation): PK-1 (**g**), PANC-1 (**h**), and MIAPaCa-2 (**i**).

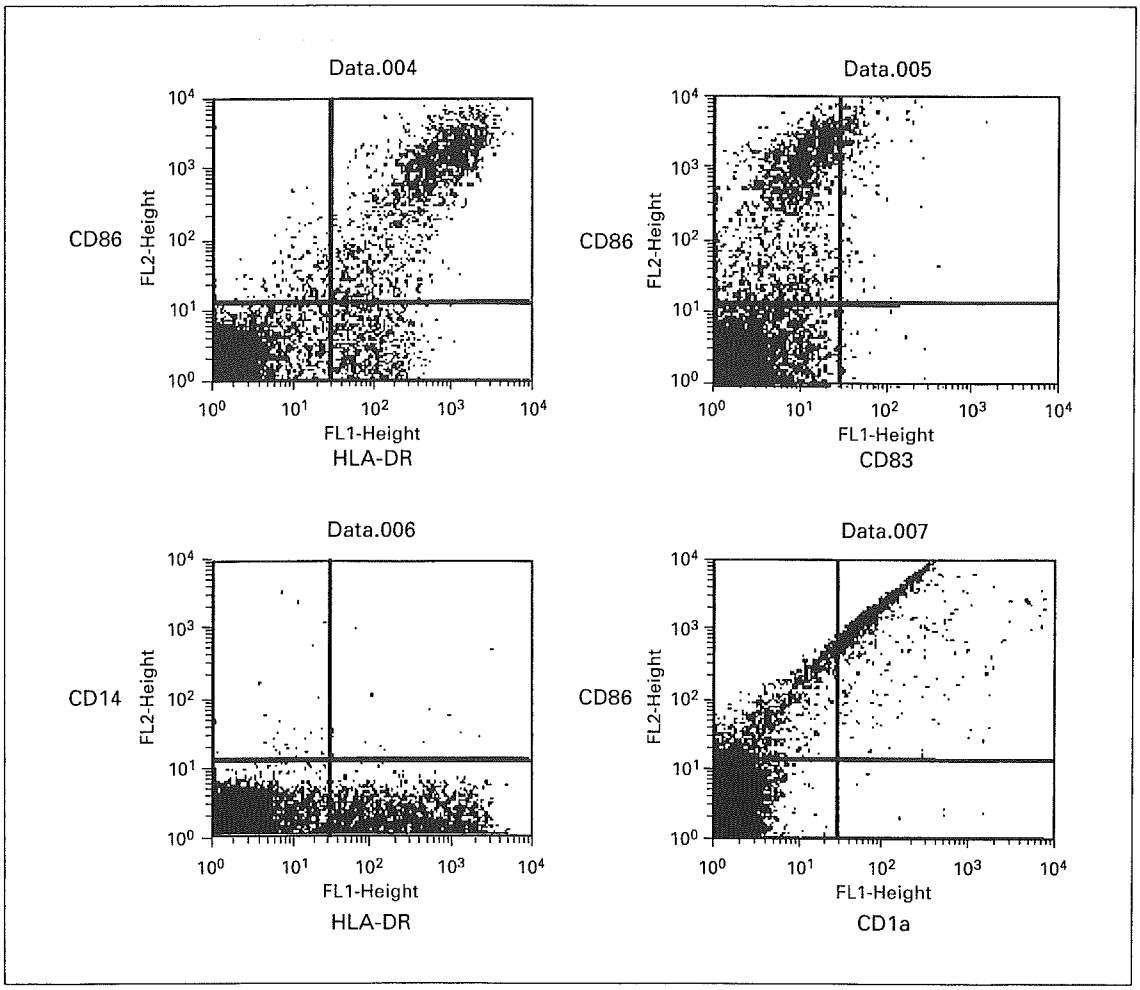

Fig. 2. Phenotype of harvested DCs.

tive) and necrosis (annexin V and PI, double positive) were detected 3 h after irradiation, and dead cells increased during the time course up to 6 h (fig. 1b, c). Next, we tested the effects of different doses of irradiation on the cell death induction. When harvested at the same time point, PK-1 irradiated at higher doses was more likely to undergo both apoptosis and necrosis (fig. 1d–f). Then, different tumor lines were used in the same experiment. All tumor cells showed both apoptotic and necrotic change to some extent. Interestingly, MIAPaCa-2 was more likely to undergo apoptosis than necrosis, while in the other two cell lines the opposite tendency was observed (fig. 1g–i).

DCs Phagocytize Irradiated Pancreatic Cancer Cells

We propagated immature DCs from PBM using GM-CSF and IL-4. The phenotype of the DCs used in this study is shown in figure 2. We defined immature DCs as CD1a-positive, CD14-negative, and CD83-negative. Contamination by lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$ or $CD19^+$) was less than 5% (data not shown).

We investigated whether these DCs could phagocytize irradiated pancreatic cancer cells using fluorescent microscopy and a flow cytometric procedure. DiI-stained MIAPaCa-2 (fig. 3a) was irradiated at 30 Gy, and put in a 6-well plate together with DiO-stained DCs (fig. 3b). These cells were co-incubated for up to 18 h, and the interaction of DCs with irradiated MIAPaCa-2 was observed under fluorescent microscopy. DCs contacting the tumor cells were observed as early as 4 h after co-incubation (fig. 3c). After 18 h, not all but many DCs were found to contain tumor-derived apoptotic bodies within them (fig. 3d). Some DCs containing apoptotic bodies showed 'dendritic processes' (fig. 3e).

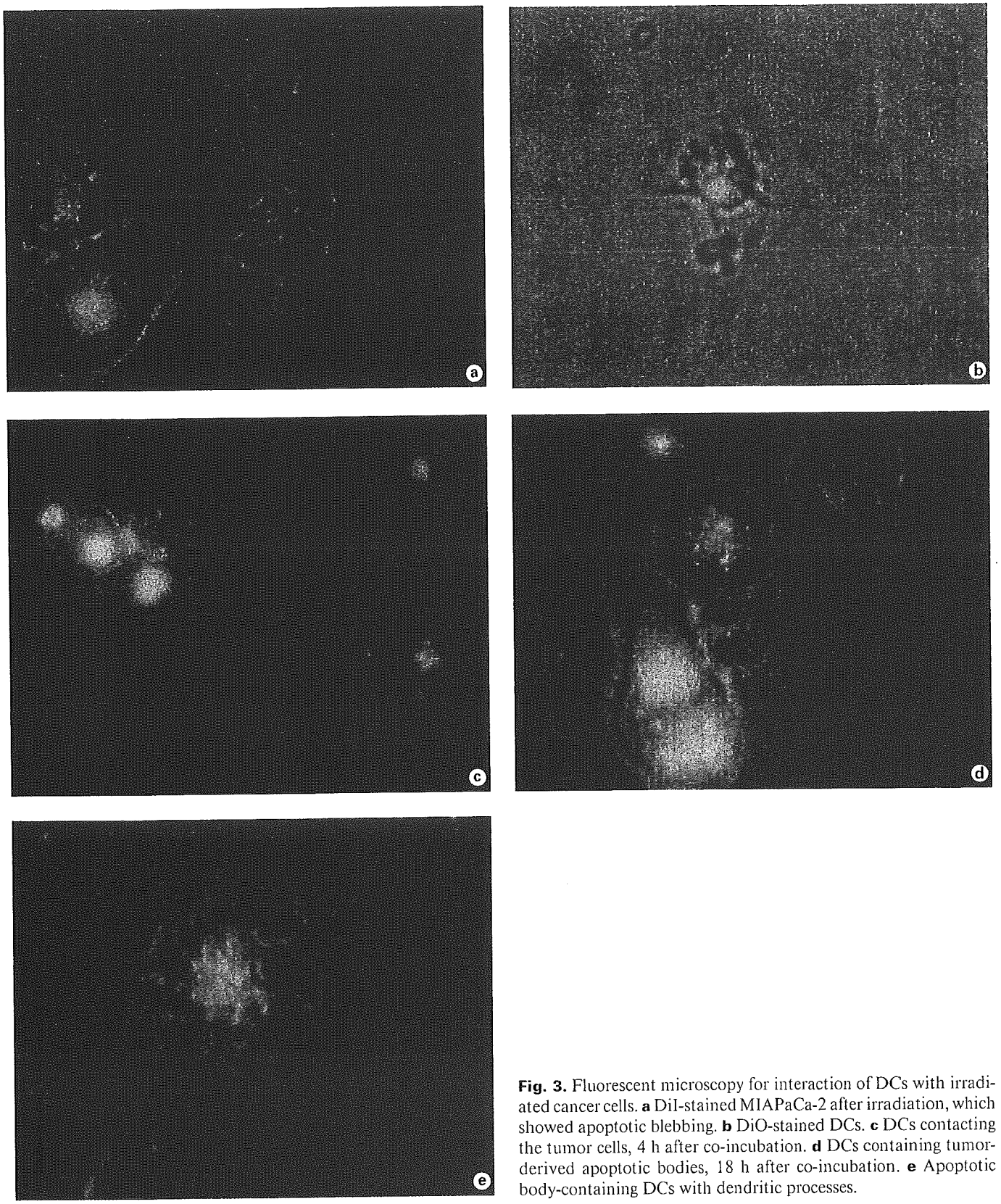

Fig. 3. Fluorescent microscopy for interaction of DCs with irradiated cancer cells. **a** DiI-stained MIAPaCa-2 after irradiation, which showed apoptotic blebbing. **b** DiO-stained DCs. **c** DCs contacting the tumor cells, 4 h after co-incubation. **d** DCs containing tumor-derived apoptotic bodies, 18 h after co-incubation. **e** Apoptotic body-containing DCs with dendritic processes.

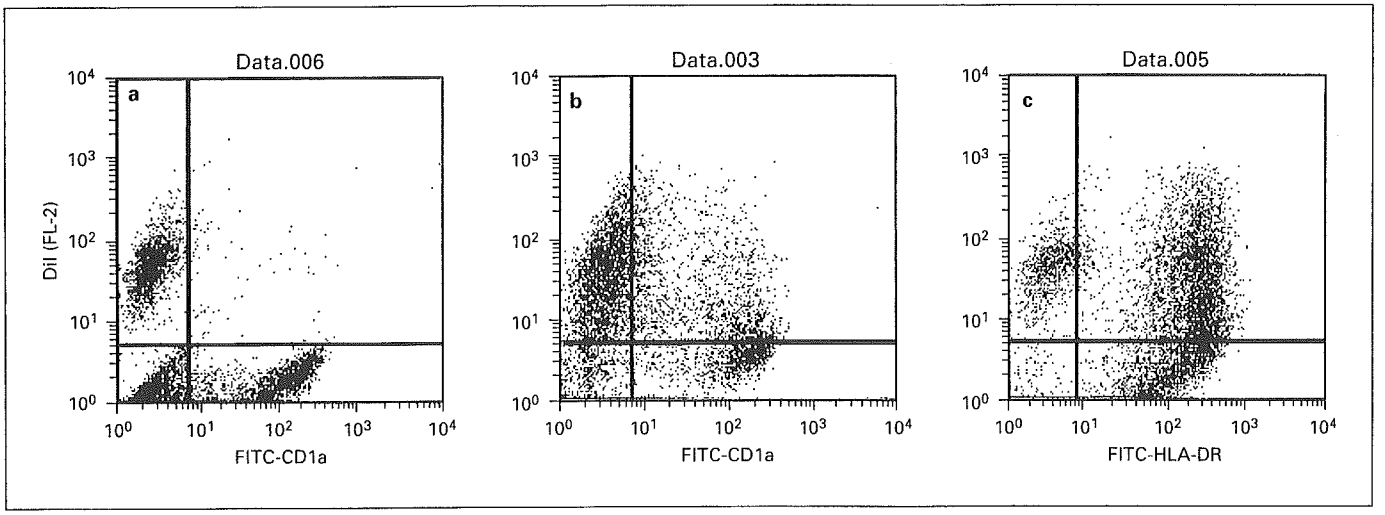

Fig. 4. Flow cytometry for interaction of DCs with irradiated cancer cells. **a** Before co-incubation. **b**, **c** 18 h after co-incubation.

To confirm the DCs' uptake of dead MIAPaCa-2, flow cytometric examination was performed. DiI-stained MIAPaCa-2 was irradiated at 30 Gy, and co-incubated in a 6-well plate with unstained DCs. After 18 h, all cells were harvested and stained with FITC-conjugated antibodies against CD1a or HLA-DR. Flow cytometric analysis revealed that most DCs ($CD1a^+$ or $HLA\text{-}DR^+$) showed positive fluorescence for DiI indicating that the DCs contained DiI-positive tumor fragments (fig. 4).

Discussion

In this study, we first examined how irradiation killed pancreatic cancer cells. Flow cytometric experiments revealed that both apoptosis and necrosis could be induced in the tumor cells by ionizing radiation. The apoptotic change observed in irradiated pancreatic cancer cells has intriguing implications. p53 is thought to be required to induce apoptosis, whereas all the tumor cells used in this study were p53-deficient cancer cell lines [16–18]. Accordingly, the apoptosis induced in irradiated tumor cells could proceed in a p53-independent manner.

DCs are able to present antigens via MHC class I as well as class II [19]. Endogenous antigens such as proteins derived from infected viruses are degraded through the ubiquitin-proteasome system in the cytosol to 8- to 9-amino-acid fragments, which are then transported into the endoplasmic reticulum, where they become associated with MHC class I molecules and are thus delivered to the cell surface [20]. On the other hand, exogenous antigens are internalized by phagocytosis or pinocytosis, processed through the endosome/lysosome system without entering the cytosol, captured by MHC class II molecules and presented on the cell surface [21]. In addition, DCs have the ability to present exogenous antigens on MHC class I, which is called 'cross-presentation' [22]. Internalized exogenous antigens are transferred to the cytosol in an undefined manner, ubiquitinated, processed by proteasome, transported into the endoplasmic reticulum and presented on MHC class I. However, the presentation of exogenous antigens on MHC class I or II may be regulated by the state of maturation of DCs [23]. For cross-presentation by DCs, apoptotic bodies were first proposed to be the best source of antigens [22], whereas later studies suggested that necrosis or inflammatory signals, but not apoptosis, was necessary to induce the maturation of DCs [24, 25]. Although further investigations are required, both apoptosis and necrosis might be indispensable for effective cross-presentation. In this sense, ionizing radiation can facilitate cross-presentation by DCs, since it can induce both types of death in pancreatic cancer cells – apoptosis and necrosis. If DCs encountered irradiated tumor cells, they would phagocytize the apoptotic cells, and the surrounding necrotic cells might stimulate DCs to maturate. We proved here that DCs could internalize irradiated pancreatic cancer cells, although we could not show that such dead cells were apoptotic or necrotic. The present study may, however, be insufficient to confirm that DCs really cross-present TAAs

derived from phagocytized tumor cells, because we could not utilize MHC-matched DCs and pancreatic cancer cells. Nevertheless, we provided some evidence suggesting that irradiated cancer cells could be used in DC-based immunotherapy against pancreatic cancer.

Clinical DC trials have already started for pancreatic cancer as well as other malignancies. Due to the lack of available TAA peptides of pancreatic cancer, several modalities, such as CEA mRNA loading [26], allogeneic tumor lysate [27] or apoptotic tumor cells [28], have been adopted as a source of TAAs in DC-based immunotherapy. Accordingly, we provide one more modality for obtaining the TAAs, i.e., by using ionizing radiation. DC-based immunotherapy combined with irradiation therapy may yield some insights for new therapies against pancreatic cancer.

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Virchows Arch (2005) 447: 794–799
DOI 10.1007/s00428-005-0039-7

ORIGINAL ARTICLE

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Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study

Received: 17 May 2005 / Accepted: 24 June 2005 / Published online: 9 August 2005

Abstract Now that more than two decades have passed since the first reports of intraductal papillary-mucinous neoplasms (IPMNs), it has become clear that IPMN consists of a spectrum of neoplasms with both morphological and immunohistochemical variations. At a meeting of international experts on pancreatic precursor lesions held in 2003, it was agreed that a consensus classification of IPMN subtypes should be established to enable a more detailed analysis of the clinicopathological significance of the variations. Based on our experience and on information from the literature, we selected representative histological examples of IPMNs and defined a consensus nomenclature and criteria for classifying variants as distinctive IPMN subtypes including gastric type, intestinal type, pancreato-

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Pancreas Research Foundation of Japan, the Gonryo Medical Foundation, and NIH SPORE (Specialized Programs of Research Excellence) in Gastrointestinal Cancer Grant CA62924.

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biliary type, and oncocytic type. These definitions can be used for further analyses of the clinicopathological significance of the variations of IPMN.

Keywords Pancreatic tumor · Intraductal papillary-mucinous neoplasm · Classification · MUC

Introduction

The first cases of intraductal papillary-mucinous neoplasm (IPMN) were reported in the 1970s and 1980s [17, 23]. In the 1990s, the term IPMN was coined, and the tumor was established as a special entity among the pancreatic neoplasms [25]. It was also found that IPMN is related to invasive pancreatic adenocarcinoma because one third of IPMNs have an associated invasive adenocarcinoma and some patients with a noninvasive IPMN subsequently develop either invasive colloid (mucinous noncystic) carcinoma or invasive ductal adenocarcinoma [5, 9, 10]. During the more than two decades that have passed since its first description, a number of reports have been published, and it has become clear that IPMNs include a spectrum of neoplasms with both morphological and immunohistochemical variations. These variations include neoplasms with tall columnar cells arranged in relatively short papillae without significant atypia, those with villous projections lined by cells with significant atypia, those with complex branching papillae containing cells with marked atypia, and those with oncocytic cells with complex papillae [2, 4, 8, 22, 30]. Several authors have proposed classification systems for the spectrum of morphologies seen in IPMNs [4, 8, 22, 30]. One such system divides IPMNs into clear-, dark-, and compact-cell types according to the density of the cytoplasm, shapes of the epithelial cells, and expression patterns of glycoproteins contained in mucin (MUCs), while another system separates IPMNs into intestinal, pancreatobiliary, and oncocytic subtypes primarily based on their architecture and cytology [4, 22, 30].

At a meeting of international experts on pancreatic precursor lesions held at the Johns Hopkins Hospital from August 18 to 19, 2003, a basic definition of an IPMN that is employed in the current study (Table 1) was worked out, and it was agreed that a consensus classification of IPMN subtypes should be established to make it easier to compare studies from different institutions and to improve patient care [14]. The purpose of this report is to establish consensus criteria for the classification of IPMN subtypes based on histological features and immunohistochemical reactivities with antibodies to specific types of mucin (MUCs).

Table 1 Definition of intraductal papillary-mucinous neoplasm of the pancreas [14]

Intraductal papillary mucinous neoplasm is a grossly visible, noninvasive, mucin-producing, predominantly papillary or rarely flat epithelial neoplasm arising from the main pancreatic duct or branch ducts, with varying degrees of duct dilatation. IPMNs usually produce a lesion greater than 1 cm in diameter and include a variety of cell types with a spectrum of cytologic and architectural atypia.

Materials and methods

Review of slides

Representative slides of candidate subtypes of IPMNs were selected from the pathology files of Tohoku University Hospital. They were chosen so as to represent a broad spectrum of the morphological variations of IPMN, based on discussions at the Baltimore meeting and including information from the literature and personal communications. The slide set consisted of ten cases. Each case consisted of four slides, one stained with hematoxylin and eosin and one each labeled for MUC1, MUC2, and MUC5AC. The slides were reviewed by the authors independently and subsequently discussed at an international meeting in Sendai, Japan (July 2004). The goal of this meeting was to establish an international consensus on the classification of IPMNs.

Immunohistochemistry

The primary antibodies employed were a monoclonal antibody to MUC1-core (clone Ma552, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, 1:100), a monoclonal antibody to MUC2 (clone Ccp58, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:600), and a monoclonal antibody to MUC5AC (clone CLH2, Chemicon International Inc., Temecula, CA, USA, 1:1000). Paraffin sections cut at 4 μm were dewaxed with xylene and hydrated with serial immersing in 100, 90, 70, and 50% ethanol solutions and distilled water. Antigen retrieval was performed by incubating slides in boiled citric acid buffer (0.18 mM citric acid, 0.82 mM sodium citrate) for 15 min in a microwave oven. Indirect immunohistochemical labeling was performed as follows: The slides were incubated with PBS containing 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. After washing with PBS, the slides were incubated with the primary antibody diluted in PBS containing 10% rabbit serum at 4°C overnight. After washing with PBS, the slides were incubated with the biotinylated rabbit anti-mouse IgG antibody solution (Nichirei, Tokyo, Japan) for 30 min at room temperature. The slides were then washed with PBS and incubated with streptavidin-horseradish peroxidase solution (Nichirei) for 30 min at room temperature. After washing with PBS, the slides were incubated with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) diluted in PBS containing 0.05% hydrogen peroxide at a final concentration in 0.2 mg/ml. The slides were washed with running tap water and counterstained with hematoxylin.

Table 2 Consensus nomenclature and criteria for classification of features of four types of intraductal papillary-mucinous neoplasm of the pancreas

Type	Mimicker	Criteria	Atypia	MUC1	MUC2	MUC5AC
Gastric	Gastric foveolae	Thick finger-like papillae, eosinophilic > basophilic cytoplasm, basally located nuclei. This type may have abundant flat areas	Mild/ low-grade	–	–	+
Intestinal	Intestinal villous neoplasm	Villous papillae, basophilic > eosinophilic cytoplasm, enlarged oval and hyperchromatic nuclei with pseudostratification. This type may show low papillae consisting of cells with amphophilic cytoplasm	Moderate or severe/ high-grade	–	+	+
Pancreatobiliary	Cholangiopapillary neoplasm	Thin branching complex papillae, moderate amphophilic cytoplasm, enlarged hyperchromatic nuclei	Severe/ high-grade	+	–	+
Oncocytic	Oncocytic neoplasm	Thick branching complex papillae with intracellular and intraepithelial lumina, abundant eosinophilic (oncocytic) cytoplasm, large round nuclei with prominent nucleoli	Severe/ high-grade	+	–	+

Results

All participants agreed that the IPMN cases provided covered representative histological variations of IPMN and that they can be subclassified into four types. In the following, the consensus criteria for the classification of IPMN subtypes based on morphological features and the immunohistochemical reactivity for MUCs (Table 2) are presented.

The gastric-type IPMN consisted of cells resembling gastric foveolae and usually showed low-grade atypia corresponding to a diagnosis of intraductal papillary-mucin-

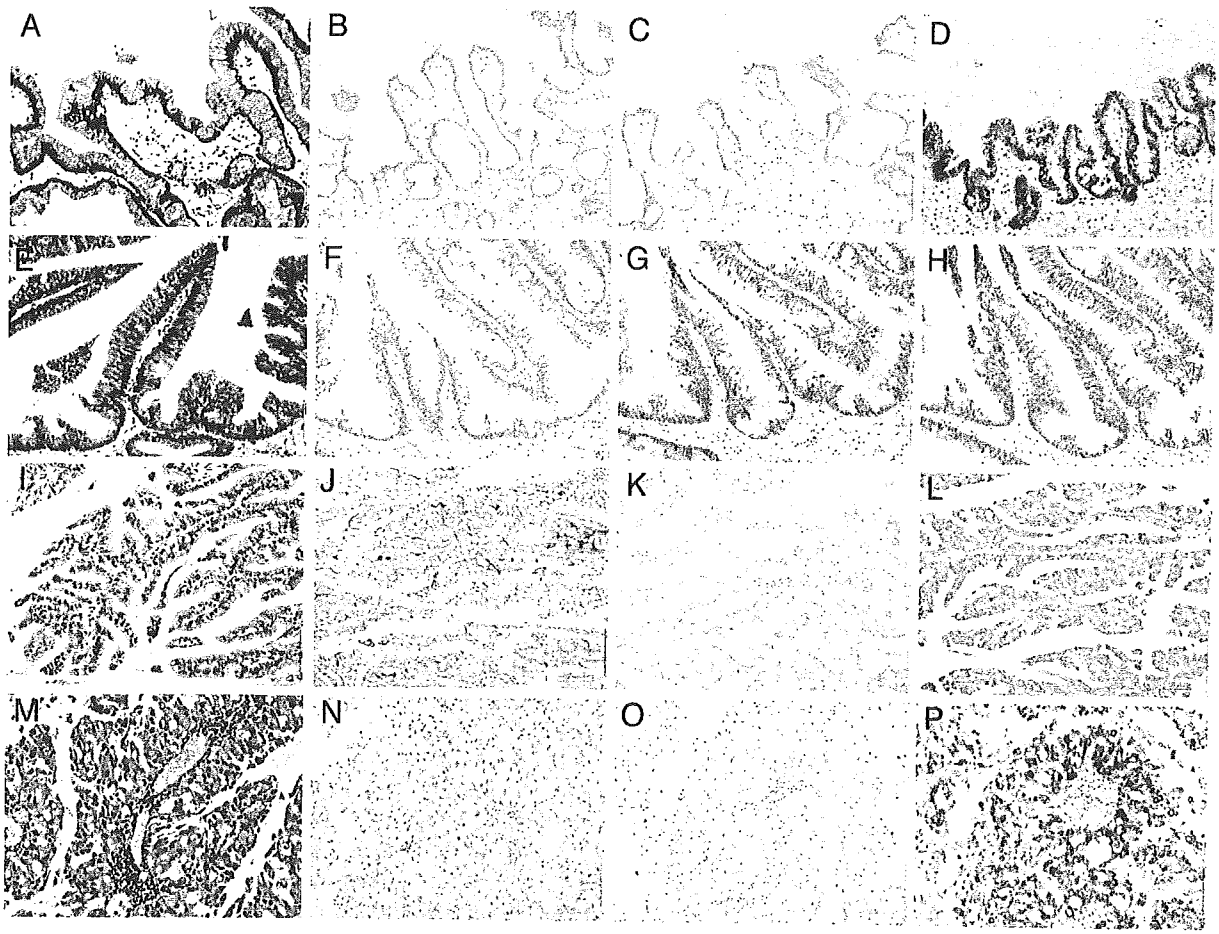

Fig. 1 Representative images of the subtypes of intraductal papillary-mucinous neoplasm of the pancreas. **a–d** The gastric type; **e–h** the intestinal type; **i–l** the pancreatobiliary type; **m–p** the oncocytic type. Hematoxylin and eosin staining (**a**, **e**, **i**, **m**) and immunohistochemical stainings of MUC1 (**b**, **f**, **j**, **n**), MUC2 (**c**, **g**, **k**, **o**), and MUC5AC (**d**, **h**, **l**, **p**)

ous adenoma (Fig. 1a). They expressed MUC5AC but were negative for MUC1 and for MUC2 (Fig. 1b–d). The gastric-type IPMN corresponded to the previously reported null type or the clear-cell type of IPMN [6, 22, 30]. The gastric-type IPMN often presented as a small cystic lesion involving branch ducts of the pancreas. This type of IPMN sometimes merged with other subtypes of IPMNs (Fig. 2c,d).

The intestinal-type IPMN resembled intestinal villous neoplasms with tall columnar epithelial cells that usually showed moderate- or severe/high-grade atypia corresponding to borderline or in situ carcinoma (Fig. 1e). It corresponded to the previously reported dark-cell type of IPMN [22, 30]. The intestinal-type IPMN usually showed large cystic lesions involving the main duct and several branch ducts. Occasionally, there were small papillae with goblet-cell-like appearance with low-grade atypia (Fig. 2a,b). The neoplastic cells consistently expressed MUC2 and MUC 5AC but were negative for MUC1 (Fig. 1f–h).

The pancreatobiliary type of IPMN consisted of cells resembling cholangiopapillary neoplasms and showed complex, thin, branching papillae with severe/high-grade atypia corresponding to carcinoma in situ (Fig. 1i). The lesion was at least focally positive for MUC1 and consistently expressed MUC5AC but not MUC2 (Fig. 1j–l).

The oncocytic type of IPMN consisted of cells with abundant, intensely eosinophilic cytoplasm and showed complex thick papillae with intraepithelial lumina and severe/high-grade atypia corresponding to carcinoma in situ (Fig. 1m). The oncocytic type of IPMN corresponded to the previously reported compact-cell type IPMN [22]. This IPMN subtype expressed MUC5AC consistently and MUC1 focally but was negative for MUC2 (Fig. 1n–p).

While it was the consensus of the group that the classification of IPMNs into these well-defined subtypes will help standardize the reporting of IPMNs, it was recognized that IPMNs often are composed of a combination of more than one cell type. The group therefore proposed that each IPMN should be subclassified by the dominant component and that any other significant subtypes present should be documented. In case of an invasive carcinoma associated with IPMN, the subtypes of the intraductal component should be evaluated and noted, especially in areas associated with the invasive component.

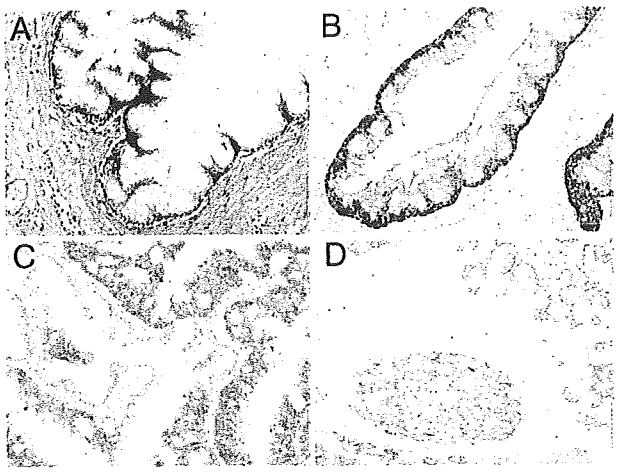

Fig. 2 Variation of the intestinal type mimicking goblet cells (hematoxylin and eosin) (**a**) and its immunohistochemical staining of MUC2 (**b**). Examples of mixed areas combining the gastric type and the intestinal type (**c**) and the gastric type and the pancreatobiliary type (**d**). Images show immunohistochemical stainings of MUC2 (**c**) and MUC1 (**d**)

Discussion

The diagnostic term IPMN encompasses a spectrum of intraductal mucin-producing neoplasms composed of a variety of cell types showing distinctive architectural and histochemical features [19, 25]. An international panel of experts was assembled to establish a nomenclature for classifying the subtypes of IPMNs. Based on the experience of the panel, information from the literature, and a review of a selected series of IPMNs, consensus criteria for the classification of subtypes of IPMNs were agreed upon. These definitions should facilitate further analyses of the clinicopathological significance of the variations of IPMN.

The panel of experts agreed unanimously that four types of IPMN can be distinguished among the series of cases that had been studied. These IPMN types were classified as gastric, intestinal, pancreatobiliary, or oncocytic type.

According to the currently available data, the gastric-type IPMN usually presents as relatively small cystic lesions in peripheral branch ducts with low-grade atypia, mostly corresponding to intraductal papillary-mucinous adenoma, while the other subtypes of IPMN usually present as large lesions involving the main duct and connecting branch ducts with marked atypia, mostly corresponding to intraductal papillary-mucinous neoplasm in the borderline category or intraductal papillary-mucinous carcinoma [4, 28]. These latter types of IPMN are often associated with an invasive adenocarcinoma [10, 18, 28].

We present this subclassification of IPMN to aid in the comparison of studies from different institutions. One interpretation of our findings is that the types of IPMN described here may simply be a manifestation of a spectrum in the differentiation and progression of a single lineage of IPMN and that these morphologies may, in part, reflect the degree of dysplasia. IPMN may be a progressive neoplastic lesion, in which a small cystic lesion with low-grade atypia may progress to large multicystic ductal lesions with severe atypia and complex histological architecture and, eventually, to invasive cancer. Areas with gastric-type epithelium can be associated with the other subtypes, implying that the gastric type might be a common precursor of the other types [6], although we feel that such a conclusion is premature. Whether the IPMN types we defined are truly distinctive lineages or variations of a single progressive neoplastic lineage should be further clarified by clinicopathological and molecular studies based on the definitions provided. One study on 51 pancreata with IPMNs that compared the various subtypes of IPMN and aberrations of tumor suppressor genes, includ-

ing CDKN2A, TP53, SMAD4, and DUSP6, revealed no significant association [11]. It is hoped that such studies will lead to an understanding of the mechanisms by which IPMNs develop and progress.

Pancreatic intraductal neoplasms consisting mainly of tubular glands of pyloric-gland type have been reported and designated as "intraductal tubular adenoma" [7, 15]. We did not include examples with predominantly tubular growth in the current study. However, because focal tubule formation is often observed in IPMNs, especially those of the gastric type or the pancreatobiliary type, the authors would assume that tubular growth may occur in IPMNs and that neoplasms with a predominantly tubular pattern may correspond to intraductal tubular adenoma, pyloric-gland type. The coexistence of papillae and tubules in both IPMNs and intraductal tubular adenoma suggests that they may be closely related, albeit distinctive, morphological features.

MUCs are useful markers for evaluating subtypes of IPMN [13, 20–22, 29, 30]. MUC1 is a component of the membrane-bound type of mucin and is usually detected in intralobular ductal lumina in the normal pancreas [1]. MUC1 is expressed in invasive ductal adenocarcinoma of the pancreas, often showing extensive positivity [24]. Among the IPMN subtypes, the pancreatobiliary type usually shows positivity for MUC1, which parallels its association with invasive ductal adenocarcinoma of the conventional tubular type [6]. The oncocytic type expresses MUC1 focally, and while it has been reported to be associated with invasive oncocytic carcinoma, its association with invasive ductal carcinoma is unknown [2]. MUC2 consists of secreted mucin usually observed in intestinal glands and is considered to be a marker of intestinal differentiation [12]. The intestinal type of IPMN, of both villous and goblet-cell features, strongly expresses MUC2 [6, 13, 21, 22, 24, 30]. The intestinal differentiation of this subtype of IPMN was supported by the demonstration that they also express CDX2, a homeobox gene involved in intestinal development [6]. MUC5AC is expressed by mucous surface cells of the stomach. MUC 5AC is not detected in the normal pancreas but is consistently detected by immunohistochemistry in all types of IPMN [13, 30]. It is also known that pancreatic intraepithelial neoplasia (PanIN), precursor lesions associated with invasive ductal adenocarcinoma, also consistently express MUC5AC [16, 21]. PanINs are usually negative for MUC2 but often express MUC1, especially in high-grade atypical lesions [5]. The differential diagnosis between PanIN and IPMN was discussed in detail in a previous publication [14].

The prognosis of patients with an IPMN has been shown to depend on the presence or absence of an associated invasive carcinoma and, if an invasive carcinoma is present, on the histological type of the associated invasive carcinoma [26]. Invasive carcinomas associated with IPMN largely consist of two distinct types, invasive carcinoma mimicking usual invasive ductal adenocarcinoma and invasive colloid (mucinous noncystic) carcinoma [5, 21]. Patients with an IPMN associated with an invasive colloid (mucinous noncystic) carcinoma usually have a better prognosis than do those with an associated invasive ductal adenocarcinoma [3, 5].

The classification proposed is based primarily on morphological features. The immunolabeling for MUCs may serve as a confirmatory marker for the classification. We believe our definitions may contribute to an understanding of the biologic behavior of IPMN and to better clinical management. For example, it would be valuable to determine whether a particular type of noninvasive IPMN is associated with a particular type of invasive adenocarcinoma. It will be also of interest to clarify the relationship of the intraductal tubular neoplasm of the pancreas [27] with the types of IPMN described herein.

Acknowledgements We thank Ms. Naomi Kanai for technical assistance and Ms. Kay Dege for editing the manuscript.

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