much larger compared with the numbers reported in former studies, suggesting that the present study findings may help establish a method of follow-up for UICC stage I patients in the future.

In most carcinomas other than colorectal carcinoma, when recurrence is discovered after resection of the primary lesion, they are treated as a systemic disease and salvage surgery is infrequently indicated for the recurrent lesion. However, in colorectal carcinoma, resection of the recurrent lesion may improve patient prognosis. In this respect, research is required to determine whether intensive follow-up for detecting recurrence earlier and initiating the treatment of it will lead to improvement in prognosis for colorectal carcinoma patients. In earlier studies, the numbers of examinations and times of the check-up conducted were different (1-13). As a matter of course, it should be recognized that with advances in technologies, the precisions diagnostic examinations are being enhanced, and new effective methods of examination are being developed. Moreover, the treatment regimens have been changing rapidly; in recent years the indications for aggressive surgical resection for recurrent lesions have been expanded, and new chemotherapies that are useful for improving patient prognosis have been identified (20-23). For the reasons mentioned above, a study that retrospectively confirms the usefulness of follow-up will not able to avoid a bias caused by the times when the study was performed.

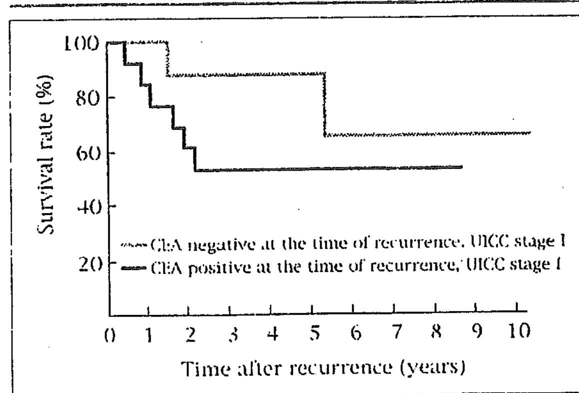

FIGURE 3a Cancer-specific survival curves after the detection of recurrence for patients who were CEA positive and CEA negative at the time of recurrence. The difference between the two groups was not significant (p=0.2734).

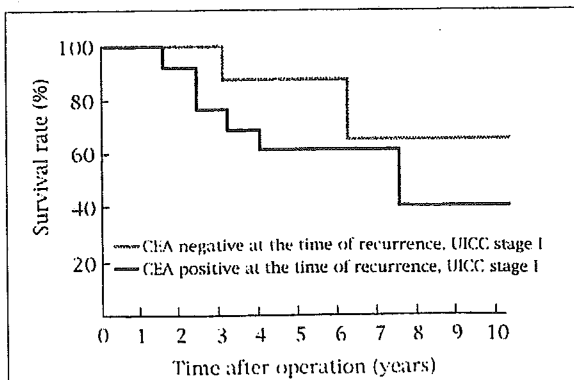

FIGURE 3b Cancer-specific survival curves after the first surgery for patients who were CEA positive and CEA negative at the time of recurrence. The difference between the two groups was not significant (p=0.3558).

TABLE 3 Site of the Primary Tumor and Recurrence

	Colon and upper/ middle rectum	Lower rectum	*P* value
Number of patients	396	145	
Recurrence			
Positive	11	10	0.0415
Negative	385	135	
Oncologic outcome			
5-Year disease-free survival (%)	97.3	92.6	0.0304
5-Year cancer-specific survival (%)	99.1	97.1	0.2402

TABLE 4 Recurrent Disease and Results of Tumor Marker Monitoring at the Time of Recurrence

Tumor marker monitoring	Elevation	No elevation	*P* value
Number of patients	13	8	
Sites of recurrence			
Liver	11	1	0.0272
Lung	2	5	
Local (Pelvis and anastomosis)	3	2	
Para-aortic lymph node	1	0	
Interval to recurrence (mo; range and median)	6-66 (19)	9-32 (18)	0.3348
Oncologic outcome 5-Year survival following first recurrence (%)	52.7	87.5	0.2734
5-Year survival after primary surgery (%)	61.5	87.5	0.3558

With regard to the value of CEA in the postoperative surveillance, some benefits have been reported from the viewpoint of earlier detection of recurrence and cost-effectiveness in detecting potentially curable recurrent disease (24-26). However, no conclusion has been reached whether the earlier detection of recurrence using CEA may influence the prognosis. In the present study, 62% (13/21) of patients with recurrence showed an increased CEA level at the time of recurrence. In these patients, the follow-up that used CEA alone might have enabled the confirmation of recurrence if diagnostic imaging was performed at the point when an increased level of CEA was recorded. However, the question here is about those cases in which recurrence was confirmed first by diagnostic imaging without showing an increased level of CEA. Of these patients, 75% (6/8) remain disease-free to date, and there is a possibility that with the follow-up using CEA alone, asymptomatic recurrences without CEA elevation may not be detected. However, these 6 patients comprised only 1.1% (6/541) of all study patients, and it may therefore be inefficient to conduct the usual postoperative surveillance while burdening the remaining 99% patients with huge costs and effort. In all UICC stage I carcinoma patients, there was a low recurrence rate of 3.9% (21/541), and in addition,

because two-thirds of recurrences could be identified using CEA, the CEA test alone may be adequate at each visit, at least for UICC stage I patients.

Another problem in the CEA examination is that encountering a patient who shows false-positivity is inevitable. Moertel *et al.* (27) reported that when the preoperative CEA level was 5ng/mL or higher, false-positivity may appear approximately in 30% of such cases. If a UICC stage I patient shows an increased CEA level during the follow-up that uses CEA alone, it may be necessary to perform examinations for other carcinoma occurrences in addition to the metastasis and recurrence of the primary colorectal carcinoma.

A noteworthy aspect of the present study was that the patients with lower rectal carcinoma showed a significantly higher incidence of recurrence. Wichmann *et al.* (19) also reported that although there was no significant difference across UICC stage I patients, rectal carcinoma involved a higher rate of recurrence, with particularly more local recurrence, compared with colon carcinoma. The CEA positive rate in patients with local recurrence of rectal carcinoma was not as high as that in patients with hepatic metastasis (2,27,28). Hence, especially in conducting follow-up examinations of patients with lower rectal carcinoma, special attention should be paid to local recurrence, and when any symptom such as pain, hemorrhage, or change in bowel habit appears, necessary examinations should be performed early.

In the present study, the UICC stage Ia group included a significantly smaller number of patients with lower rectal carcinoma. This may be because some patients who had pT1 carcinoma at the lower rectum were followed up after undergoing trans-anal resection alone. The treatment of T1 and T2 carcinoma of the lower rectum is controversial, and several studies have suggested satisfactory tumor control after local excision for lower rectal T1 and T2 carcinoma (29,30). However, recent studies suggested that local excision of T1 and T2 rectal carcinoma is followed by a much higher recurrence rate than previously reported (31,32). In our institution, a radical surgery of low anterior resection or abdominoperineal resection is often indicated for T2 lesions and most T1 lesions with adverse risk factors, especially poorly differentiated carcinoma, lymphovascular invasions, incomplete excision, or massive invasion of carcinoma to the submucosal layer. Although most patients with T1 and T2 carcinoma lesions in the lower rectum in whom local recurrence develops after local excision can be salvaged by radical resection, the long-term outcome remains unknown (33).

In the field of the postoperative follow-up examination, the value of colonoscopy has been discussed. Periodic colonoscopy may be useful for detecting anastomotic and locoregional recurrences after colorectal carcinoma operation in addition to finding metachronous colorectal carcinoma (34,35). However, in UICC stage I patients, the anastomotic and locoregional recurrences have involved a very low proportion of 1% to 3%, according to previous and the present study (19). Particularly in patients with colonic carcinoma, there have been no anastomotic or locoregional recurrences observed at our institution. Performing colonoscopy is not warranted for the purpose of detecting anastomotic and locoregional recurrences in UICC stage I patients.

In conclusion, for UICC stage I patients, the incidence of recurrence was lower, and it is therefore possible to reduce the times and screening examinations for the postoperative surveillance. Regarding screening examinations, the CEA measurement every six months until two years after the operation, and subsequently once per year until the 5th postoperative year appears to be sufficient. Nevertheless, for patients with UICC stage Ib disease and those with lower rectal carcinoma, oncologists need to pay special attention because the rates of recurrence are significantly higher.

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Original Paper

Transcatheter Management for Multiple Liver Tumors after Hepatic Artery Obstruction Following Reservoir Placement

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KEY WORDS:
Liver neoplasms; Hepatic artery; Obstruction; Reservoir; Treatment

ABBREVIATIONS:
Gastroduodenal Artery (GDA); Posterior Superior Pancreaticoduodenal Artery (PSPDA); Right Gastric Artery (RGA); Hepatocellular Carcinoma (HCC); Transcatheter Arterial Infusion (TAI); Transcatheter Arterial Chemoembolization (TACE); Dorsal Pancreatic Artery (DPA); Inferior Phrenic Artery (IPA); Inferior Thoracic Artery (ITA); Superior Mesenteric Artery (SMA)

ABSTRACT

Background/Aims: Hepatic arterial infusion chemotherapy via an implantable port system has been widely used to treat unresectable liver neoplasms. Complications of the hepatic artery occlusion following reservoir placement, however, makes it impossible to continue the infusion therapy. The purpose of our study was to assess the possibility of transcatheter treatment after the hepatic artery obstruction following reservoir placement.
Methodology: Between April 1999 and May 2002, 14 patients with liver tumors had the complication of hepatic artery obstruction following reservoir placement. We conducted a prospective trial to assess 1) the collateral pathways of feeding artery using angiography, 2) the possibility of transcatheter treatment or 3) re-reservoir placement for liver tumors.
Results: 1) Angiography revealed that the main collateral pathway of the feeding artery was the inferior phrenic artery in 7 patients (50%), the dorsal pancreatic artery in 4 patients (29%) and the anastomotic branch of the celiac axis in 1 patient (7%). The main collateral pathway could not be detected in 2 patients (14%). 2) Transcatheter treatment was successfully performed in all patients (100%). 3) Re-reservoir placement failed in all cases.
Conclusions: These results suggest that transcatheter treatment may be possible for patients with hepatic artery obstruction.

INTRODUCTION

For the last ten years, hepatic arterial infusion chemotherapy via an implantable port system has been widely used to treat unresectable malignant neoplasms of the liver (1-8). It has the advantage of increased local drug concentration, resulting in a high tumor response rate, with less systemic toxicity (1-8). However, it often causes occlusion of the hepatic artery; rates of occurrence of 10-40% have been reported (9-11).

Concerning the procedures of reservoir placement, aberrant hepatic arteries are usually embolized to permit global perfusion of the liver via a single hepatic artery that depends on development of the intrahepatic collateral circulation, and also the gastroduodenal artery (GDA) including the posterior superior pancreaticoduodenal artery (PSPDA) is embolized for preventing leakage of anticancer drug to the upper gastrointestinal region. The right gastric artery (RGA) is also embolized as much as possible. The complication of hepatic artery occlusion following reservoir placement makes it impossible to continue the infusion therapy.

We conducted a prospective trial to assess the collateral pathways of feeding artery to liver tumors and the possibility of transcatheter treatment for liver tumors after obstruction of the hepatic artery following reservoir placement.

METHODOLOGY

Between April 1999 and May 2002, 74 patients with liver tumors underwent reservoir placement. Twenty-one of the 74 patients had the complication of hepatic artery obstruction during hepatic arterial infusion chemotherapy via an implantable port system (n=18) or during procedures for performing reservoir placement (n=3). Fourteen of 21 patients resulted in tumor response (n=11) or had a complication of the procedures (n=3), and the subjects of our study were these 14 patients (eight men, 6 women; age range, 42-74 years; mean age, 61 years) with multiple liver tumors [5 hepatocellular carcinomas (HCCs), 9 metastatic tumors: 3 from colorectal cancer, 2 from gastric cancer, 2 from breast cancer, 1 from lung cancer and 1 from uterine cervical cancer] in whom we tried to perform transcatheter treatment again.

As shown in **Table 1**, of the 14 patients in this study, the GDA and the PSPDA were embolized in all patients, and the RGA was embolized in 12 patients during the reservoir placement. Four patients who had an aberrant hepatic artery underwent embolization of the aberrant hepatic artery.

Routine angiography was performed by using a 5-F pigtail catheter for abdominal aortic angiography, a

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TABLE 1 Embolization Sites of Arteries during Reservoir Placement

Patient No./ Age(y)/Sex	Primary	Occlusion of hepatic artery	Embolization site of Arteries for implantable port			
			GDA	PSPDA	RGA	Aberrant A
1/66/M	Lung ca.	CHA#	Done	Done	Done	None
2/62/M	Colon ca.	CHA-PHA‡	Done	Done	Done	Done
3/69/F	Gastric ca.	CHA-PHA	Done	Done	Done	None
4/66/M	Colon ca.	CHA-PHA	Done	Done	None	None
5/42/F	Breast ca.	CHA	Done	Done	Done	Done
6/52/F	Uterine cervical ca.	CHA	Done	Done	Done	Done
7/70/F	Gastric ca.	CHA-PHA	Done	Done	Done	None
8/50/F	Breast ca.	CHA	Done	Done	Done	None
9/74/M	Colon ca.	CHA-PHA	Done	Done	Done	None
10/51/M	HCCs	CHA-PHA	Done	Done	Done	None
11/67/F	HCCs	CHA-PHA	Done	Done	None	Done
12/67/M	HCCs	CHA-PHA	Done	Done	Done	None
13/60/M	HCCs	CHA-PHA	Done	Done	Done	None
14/68/M	HCCs	CHA-PHA	Done	Done	Done	None

#common hepatic artery; ‡proper hepatic artery.

TABLE 2 Collateral Pathways to the Tumors and Transcatheter Treatment

Patient No./ Age(y)/Sex	Primary	Occlusion of hepatic artery	Involvement of Arteries					TAE or TAI
			IPA	ITA	DPA	Periportal	Others	
1/66/M	Lung ca.	CHA#	Negative	Negative	Positive*	Negative	Negative	TAE
2/62/M	Colon ca.	CHA-PHA‡	Negative	Negative	Positive*	Negative	anastomotic br.	TAI
3/69/F	Gastric ca.	CHA-PHA	Negative	Right	Positive*	Positive	Negative	TAE
4/66/M	Colon ca.	CHA-PHA	Right	Negative	Positive	Positive	Negative	TAI
5/42/F	Breast ca.	CHA	Negative	Right	Negative	Positive	Anastomotic br.*	TAE
6/52/F	Uterine cervical ca.	CHA	Negative	Negative	Positive*	Positive	Negative	TAE
7/70/F	Gastric ca.	CHA-PHA	Right*	Negative	Positive	Positive	Duodenal br.	TAE
8/50/F	Breast ca.	CHA	Right	Negative	Negative	Positive	Negative	TAI
9/74/M	Colon ca.	CHA-PHA	Right*	Negative	Negative	Negative	Negative	TAE
10/51/M	HCCs	CHA-PHA	Bilateral*	Negative	Negative	Negative	Negative	TAE
11/67/F	HCCs	CHA-PHA	Right*	Negative	Negative	Negative	Negative	TAE
12/67/M	HCCs	CHA-PHA	Right*	Negative	Negative	Positive	Negative	TAE
13/60/M	HCCs	CHA-PHA	Right*	Negative	Negative	Negative	Negative	TAE
14/68/M	HCCs	CHA-PHA	Right*	Negative	Negative	Positive	Negative	TAE

#common hepatic artery; ‡proper hepatic artery, *main feeding artery.

5-F shephard hook catheter for celiac, superior mesenteric and inferior phrenic arteriography and a 5-F headhunter catheter for internal thoracic arteriography. When the 5-F catheter could not be inserted into the targeted artery, a 0.016-inch guidewire (Transend TM EX, Boston Scientific TARGET, US) was inserted into the targeted artery, and a 2.3-F microcatheter (Rapid Transit, Cordis, US) was advanced to it along the guidewire.

After performing routine angiography, tumor vessels, tumor stains and feeding arteries were evaluated by two radiologists (S.M., Y.A.). After reaching a consensus regarding the feeding arteries to liver tumors, we tried to advance a microcatheter to them for treatment.

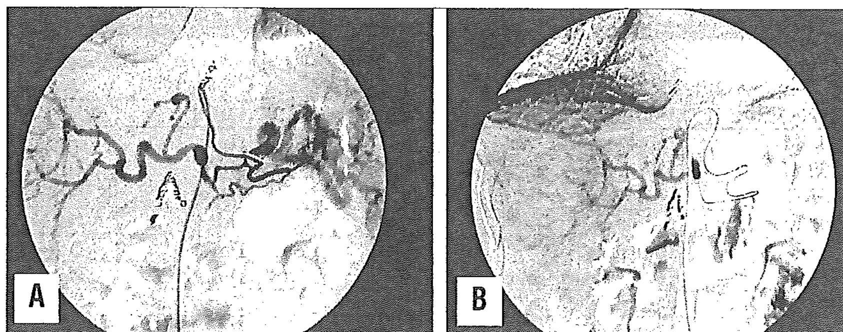
Concerning transcatheter treatment, transcatheter infusion therapy (TAI) was performed in cases in which the periportal collateral arteries were the main feeding arteries because of preventing side effects such as cholangitis or gastrointestinal damage caused by embolization, or in the cases in which the main feeding artery was not revealed. Transcatheter arterial chemoembolization (TACE) with iodized poppy seed oil for HCCs or Amilomer (Spherex, Yakult Honsha Co.Ltd., Japan) with mitomycin C and contrast material for metastatic liver tumors was performed in the remaining cases. Then, when a microcatheter was advanced into the hepatic artery, we tried to place the long tapering type catheter (Anthron P-U catheter, Toray Medical Co., Ltd., Japan, Piolax W Spindle catheter, Piolax Medical Devices, INC., Japan) for an implantable port system. Informed consent was obtained from all patients.

RESULTS

These results are shown in **Table 2**.

Angiography revealed that: the main collateral pathway of the feeding artery was the inferior phrenic artery (IPA) in 7 of 14 patients (50%), the dorsal pancreatic artery (DPA) in 4 of 14 patients (29%) and the

FIGURE 1
A 66-year-old man with liver metastases from lung cancer. **(A)** Arteriography via the DPA demonstrates the proper hepatic artery via the anastomosis of the DPA. **(B)** A microcatheter is advanced to the proper hepatic artery via the anastomosis of the DPA, and arteriography shows the global perfusion in the liver. We tried to change the microcatheter for the long tapered type catheter, but it failed because the long tapered type catheter could not be inserted into the anastomotic branch between the DPA and the proper hepatic artery.



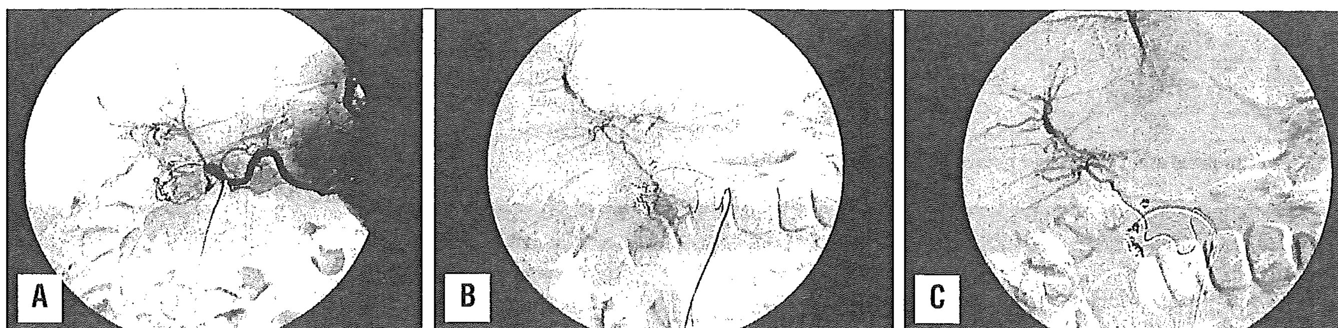


FIGURE 2 A 62-year-old man with liver metastases from colon cancer. **(A)** Celiac arteriography shows the common hepatic and the proper hepatic artery occlusion, and opacified intrahepatic collaterals. The catheter (Anthron PU catheter) is placed in the GDA for reservoir placement. **(B)** Angiography via the DPA demonstrates the intrahepatic collaterals via the pancreas arcade. **(C)** A microcatheter is advanced into the collateral artery near the intrahepatic arteries, and arteriography shows the intrahepatic arteries, not collaterals to the gastrointestinal area.

anastomotic branch of the celiac axis in 1 of 14 patients (7%). The main collateral pathway could not be detected in 2 of 14 patients (14%). Transcatheter treatment was successfully performed in all patients (100%). TACE was performed in eleven of 14 patients (79%) and TAI in the remaining 3 patients.
These results were shown in detail as follows:

Liver Metastases (n=9)

1) Collateral pathways of feeding artery

Main feeding arteries: the DPA **(Figures 1 and 2)** was in 4 of 9 patients, the right IPA was in 2 of 9 patients **(Figure 3)** and the anastomotic branch via the celiac axis was in 1 of 9 patients. The main collateral pathway could not be detected in 2 of 9 patients.

Other feeding arteries: the DPA was in 2 patients, the right IPA was in 2 patients, the right internal thoracic artery (ITA) was in 2 patients, the periportal collaterals was in 6 patients, the anastomotic branch via the celiac axis was in 1 patient and the duodenal branch in 1 patient.

2) Transcatheter treatment

Six of 9 patients successfully underwent TACE via the DPA in 3 patients, via the right IPA in 2 patients and via the anastomotic branch through the celiac axis in 1 patient. In the cases with TACE performed via the DPA and via the anastomotic branch, it was possible to advance a microcatheter into the proper hepatic artery (n=2) or the right hepatic artery (n=1) or the left hepatic artery (n=1) through the DPA or the

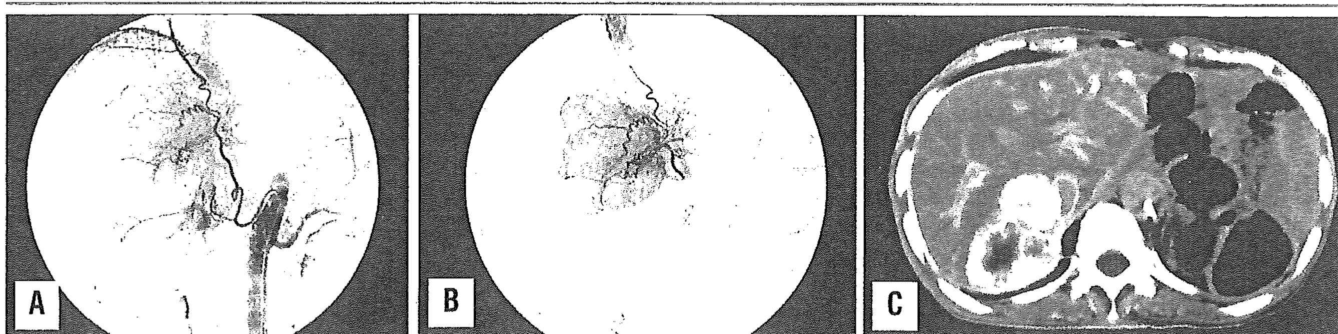


FIGURE 3 A 70-year-old woman with liver metastases from gastric cancer. **(A)** Selective right IPA arteriography reveals that the right IPA feeds the liver metastases. **(B)** A microcatheter is advanced to the distal site of the right IPA and TAE was performed via this site using Amilomer with mitomycin C and contrast material. **(C)** Nonenhanced CT image obtained after TAE shows accumulation of contrast material in the tumor.

anastomotic branch. In the remaining 3 patients, TAI was performed via both the DPA and the right IPA in 1 patient, via both the right IPA and the periportal collaterals in 1 patient and via the DPA in 1 patient. There were no complications related to TACE and TAI.

3) Re-reservoir placement

We tried to advance the long tapered type catheter for reservoir placement to one of the hepatic arteries via the collateral pathway in 4 cases, but it failed in all cases.

Hepatocellular Carcinomas (n=5)

1) Collateral pathways of feeding artery

Main feeding artery: the right IPA was in 4 patients and the bilateral IPAs were in 1 patient.

Other feeding arteries: the periportal collaterals were in 2 patients.

2) Transcatheter treatment

TACE was successfully performed only via the IPA in 4 patients and via the bilateral IPAs in 1 patient. Three of 5 patients had a complication of pleural effusion after TACE. However, pleural effusion diminished within 4 weeks in all 3 patients.

DISCUSSION

Obstruction of the hepatic artery after reservoir placement has been one of the most difficult problems of infusion chemotherapy. In this situation, the pancreaticoduodenal arcade thought to become a feeding artery in cases of obstruction of the common hepatic artery had been embolized already and also the aberrant hepatic artery had been embolized. Therefore, there are few pathways for advancing a catheter into the feeding artery to the tumors. Generally, systemic chemotherapy is the next step of treatment for liver tumors following such an obstruction of the hepatic artery. To our knowledge, there were no reports of transcatheter treatment in cases of obstruction of the hepatic artery after reservoir placement.

It is well known that the IPAs often play a role as feeding arteries in the patients with liver tumors adjacent to the liver surface (12,13). When HCCs are located in the ventral hepatic areas directly beneath the diaphragm, the ITAs serve as feeding arteries after hepatic artery occlusion caused by repeated TACE (14). Takeuchi *et al.* reported that the liver was supplied by the IPAs, by the superior mesenteric artery (SMA), by the celiac axis and the left gastric artery under temporary balloon occlusion of the proper hepatic artery (15). In our present cases, the GDA, the PSPDA and the RGA were already embolized by coils. Then, the SMA and the left gastric artery might be thought to become a feeding artery with lower frequency.

Our present study revealed that the IPAs serve as feeding arteries in the patients with HCCs and metastatic liver tumors as some researchers reported, but it was noted that the DPA had a tendency to be the feeding artery in cases of metastatic liver tumors. Before starting this study, we thought that the ITAs might be one of the main feeding arteries. There was no case that the ITA was the main feeding artery. This result could be disputed due to the fact that the time from initial TACE to ITA angiography was too short (ranged from 0 to 7 months, mean; 3.1 months) to develop anastomoses between the hepatic arteries and the ITAs.

Llovet *et al.* performed a randomized controlled trial in patients with unresectable hepatocellular carcinomas to assess the survival benefits of regular repeated TACE compared with conservative treatment and they obtained the results that TACE induced objective responses sustained for at least 6 months, and was associated with a significantly lower rate of portal vein invasion than conservative treatment (16). TACE should be performed for improvement of survival probabilities even though the hepatic artery is occluded following reservoir placement. In our present study, TACE was successful performed via the IPAs in all five cases with HCCs.

Concerning liver metastases from colorectal cancer, survival analyses showed a significant advantage for infusion chemotherapy compared with systemic chemotherapy by randomized controlled trial studies (3-5,7,8). On the other hand, there is no definite evidence of survival benefits in patients with liver metastases from other organs. We believe, however, that TACE or TAI may be effective in treatment even for liver metastases from other organs such as breast cancer, gastric cancer etc.

In our study, 6 of 9 patients had metastatic liver tumors and TACE was successfully performed via the DPA in 3 patients, via the right IPA in 2 patients and via the anastomotic branch through the celiac axis in 1 patient. It was noted that a microcatheter was advanced into the hepatic artery through the DPA. There has been no reports of TACE or TAI being performed via the DPA for liver tumors.

Though we tried to advance the long tapered type catheter for reservoir placement to one of the hepatic arteries via the collateral pathway in 4 cases, it was impossible in all cases. If the catheter for reservoir placement is developed as the same quality of a microcatheter, reservoir placement could be successfully performed again after occlusion of the hepatic artery following reservoir placement.

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ORIGINAL PAPER

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Reduction of drug leakage by negative-balance isolated pelvic perfusion: correlation between leakage and in-out flow rate in a pig model

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Abstract *Purpose*: Isolated pelvic perfusion (IPP) therapy exposes target tissues to high doses of anticancer drugs with low systemic concentrations, but the major drawback is drug leakage into the systemic circulation, which often thwarts the increased drug concentration. In this study, the efficacy of altering the in-out flow rate during IPP in order to decrease the leakage was assessed in adult pigs. *Methods*: The abdominal aorta and the infrarenal vena cava were occluded with two balloon catheters, blood in the extracorporeal circuit was circulated with twin rotary pumps, and the IPP was performed with platinum. Three sets of in-out flow rates were used, and the degree of drug leakage into the systemic circulation was evaluated. The volume of blood withdrawn was equal to the volume returned (300 ml/min; group A), 5% higher (group B), or 10% higher (group C). The platinum concentrations in the pelvic circulation, systemic circulation, and urine were measured and compared. *Results*: The average and maximum plasma platinum concentrations in the pelvic circulation did not significantly differ among the three groups. The plasma platinum concentrations in the systemic venous circulation of the three groups significantly ($P<0.01$) decreased as the volume withdrawn during IPP increased. The percentage of platinum eliminated in the urine during IPP was significantly ($P<0.01$) lower in group B and C than in group A. *Conclusions*: Setting the volume withdrawn higher than the volume returned decreased leakage into the systemic circulation under isolated pelvic perfusion.

Keywords Isolated pelvic perfusion · Negative-balance isolated pelvic perfusion · Pig animal model

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Introduction

Patients with advanced cancer require effective treatment, including surgical resection, radiotherapy, and chemotherapy (Lingareddy et al 1997; Wong et al 1998), but only a few options are available for the treatment of advanced pelvic cancer. The first regional perfusion technique ever demonstrated permitted regional delivery of a drug at higher concentrations than conventional systemic therapy (Creech et al 1958). Isolated pelvic perfusion (IPP) was first developed by Austen et al 1959, and it has been used by several medical groups in an effort to control advanced malignancies of the bladder, uterus, and rectum (Austen et al 1959; Watkins et al 1960; Stehlin et al 1960; Shingleton et al 1961; Lawrence et al 1961, 1963; Collins 1989; Wile et al 1985, 1987; Turk et al 1993; Wanebo et al 1996, 1999; Guadagni et al 1998). IPP is the only regional perfusion technique that permits delivery of a drug into the pelvic circulation at a higher concentration than in the systemic circulation.

Isolated pelvic perfusion has recently been adopted as preoperative therapy for advanced pelvic malignancy. However, less emphasis has been placed on IPP for the past 20 years, because leakage of anticancer drugs from

the pelvic region into the systemic circulation often causes adverse side effects in the kidneys or other organs. In addition, highly-developed collateral vessels in the pelvis also contribute to such leakage, and although minor modifications of the IPP technique have been made over the past several decades in an attempt to decrease the extent of leakage, no major effective improvements have been reported. In this study, we hypothesized that drug leakage into the systemic circulation would be decreased by reducing the blood pressure in the pelvic venous circulation during IPP, and for this purpose we developed a twin-pump system for the extracorporeal circuit to modulate the in-out flow rate in an IPP pig model.

We devised a novel IPP technique that allowed control of the negative-balance in-out flow rate, which we refer to as negative-balance isolated pelvic perfusion (NIPP), and found that this technique clearly reduced the drug leakage into the systemic circulation.

Materials and methods

Animal model and general anesthesia

All animal experiments were conducted in accordance with the Guidelines of Nippon Medical School University for Animal Care and Experimentation. Fifteen adult male pigs weighing 36–42 kg (average 38 kg) were used in this study, and all procedures were performed on the animals under general anesthesia. The animals were placed in the supine position. General anesthesia was induced with an intramuscular injection of ketamine hydrochloride (300 mg/pig) and maintained with sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan).

Monitoring the systemic circulation

Peripheral arterial oxygen saturation was maintained above 95% and was monitored with a probe applied to the ear. Each animal was continuously monitored during the procedure using electrocardiography. Both internal jugular veins were exposed through a cut-down incision, and two cannula sheaths (5 Fr.; Medikit Co. Ltd.) were inserted into each of them to collect blood samples and to monitor central venous pressure (CVP). The thyrocervical or brachial artery was exposed, and a 5-Fr. cannula sheath was inserted to monitor blood pressure during the procedure. CVP and arterial blood pressure were recorded before and 0, 5, 10, 15, 20, 25, and 30 min after the start of perfusion.

Catheter technique and procedure

Both common femoral arteries and veins were exposed through a cut-down incision, and sheaths (6 Fr. and 9 Fr. each; Medikit Co. Ltd. Tokyo, Japan) were inserted into each of them, as shown in Fig. 1. The cannulas had specially designed side arms to allow high flow and to keep the pressure in the pump-system low during withdrawal and return of the blood through the cannulas (Fig. 1). After systemic heparinization (120 U/kg), the abdominal aorta and the infrarenal vena cava (IVC) were occluded with two balloon catheters (30 mm balloon with 5 Fr. shaft for the aorta, 40 mm balloon with 6 Fr. shaft for the IVC, Forte Co. Ltd., Tokyo, Japan) at the level of the L3/4 intervertebral space. Blood was withdrawn from the veins with one of the rotary pumps and returned to the arteries through the cannulas with the other rotary pump.

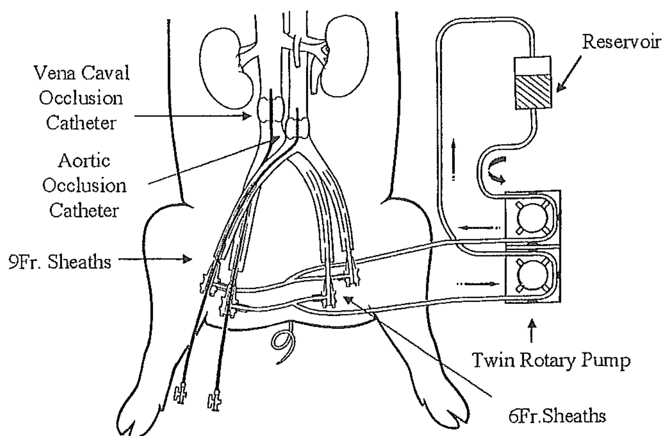

Fig. 1 Schema of the negative-balance isolated pelvic perfusion system used in the pig model

Angiography

Before IPP, isolated pelvic angiography was performed with the twin rotary pumps in each animal ($n=15$) to confirm that the IPP system was established (Fig. 2). Isolated pelvic angiography was performed by infusion of nonionic contrast material (300 mg/ml; iodine) at a rate of 5 ml/s through the arteries with a rotary pump and was withdrawn at a rate of 5 ml/s through the veins with the other rotary pump. The duration of angiography was 30 s, and the total volume of contrast material infused was 100 ml. Recording of the fluoroscopic images on video tape was begun at the start of contrast material infusion. IPP was performed after confirming that no contrast material had entered the inferior vena cava after the occluding balloon.

Experimental groups and IPP procedure

We evaluated the degree of drug leakage into the systemic circulation by using three sets of in-out flow rates and changing the volume withdrawn during IPP. Five animals were allocated to each group, and the volume withdrawn was equal to the volume returned (300 ml/min; group A), 5% higher (315 ml/min; group B), or 10% higher (330 ml/min; group C). The

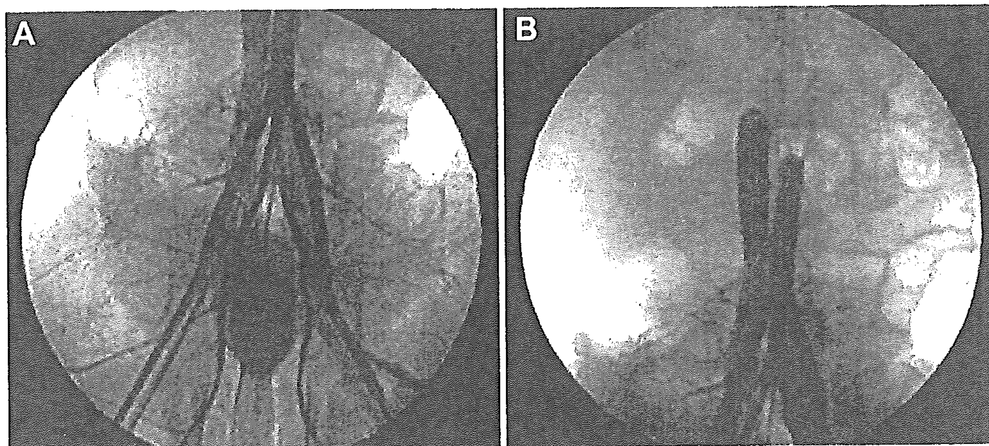

Fig. 2A–B Isolated pelvic angiography in a pig model. **A** Angiogram of the pelvis 10 s after the start of injection. **B** Angiogram of the abdomen 20 s after the start of injection. Fluoroscopic images with pelvic isolation were acquired in the pelvic cavity and in the abdomen. No contrast material entered the inferior vena cava after the occluding balloon

animals were administered the other agent by intravenous drip infusion into an ear vein during the IPP, because the reduction of blood volume caused by increasing the volume withdrawn would cause anemia in the pigs. In view of the volume of blood loss during IPP, the intravenous drip infusion rate was set at 0 ml/min in group A, 15 ml/min in group B, and 30 ml/min in group C. The platinum-containing anticancer drug nedaplatin (Shionogi Co. Ltd., Osaka, Japan), was infused for 30 min. A dose of 200 mg/pig was administered into the reservoir (Fig. 1) in three equal boluses, every 10 min at and after the beginning of perfusion. All results were performed independently five times and normalized to the body weights of the pigs, respectively.

Analysis of pharmacokinetics

Plasma platinum concentration was measured in blood samples collected from the arterial and venous sides of the pump and from the systemic venous circulation (the superior vena cava) at 0, 5, 10, 15, 20, 25, and 30 min after the start of IPP. Blood samples were collected 10 min and 20 min prior to the second and third injection of nedaplatin. Blood samples were also collected from the reservoir after perfusion. Urine was collected throughout the isolation period, and the total urine volume was recorded and used for drug analysis.

Statistical analysis

The plasma and urine samples were digested with nitric acid for analysis of metal species. Platinum concentrations in the serum and urine were measured by atomic absorption spectrophotometry (Varian SpectrAA 300/400). Drug exposure was measured as the area under the serum concentration–time curve until 30 min after the start of perfusion. All data are shown as the means ± SD. Results were compared by Tukey's studentized range test and Student's *t* test. Differences at $P < 0.05$ were considered statistically significant.

Results

Effect of IPP on the systemic circulation

Hemodynamic parameters, arterial blood pressure (BP), heart rate (HR), blood oxygen saturation (SAT), and CVP were measured during IPP to evaluate its effect on the systemic circulation. As shown in Table 1, no statistically significant differences were found in any of the parameters during IPP and NIPP, and each animal was hemodynamically stable throughout all of the processes.

Pharmacokinetics of the serum platinum concentration in the pelvic circulation

There were no significant differences in the average (based on the serum concentration–time relationship from 0 min to 30 min after the start of drug perfusion) or maximum plasma platinum concentrations in the pelvic circulation on the arterial or venous sides, and there were no significant differences between the three groups (Table 2; Fig. 3).

On the other hand, both the average and end-point plasma platinum concentrations in the systemic venous circulation decreased as the volume withdrawn during IPP increased. Significant differences in the plasma platinum concentration were observed between groups A and B, A and C, and B and C (Fig. 4). The mean ratio of pelvic platinum exposure to systemic platinum exposure was 8.9:1.0 in group A, 17.9:1.0 in group B, and 75.9:1.0 in group C, and the differences ($P < 0.01$) were significant between groups A and C and between groups B and C.

The percentage of platinum eliminated in the urine during IPP was 5.2, 0.6, and 0.5% in groups A, B, and C, respectively. The differences ($P < 0.01$) between groups A and B, and between groups A and C were significant, but the difference between groups B and C was not. The percentage of platinum remaining in the reservoir after IPP was 4.2, 12.2 and 21.4% in groups A,

Table 1 Systemic hemodynamics during isolated pelvic perfusion (IPP) and negative-balance isolated pelvic perfusion (NIPP)

	Pre-IPP	0 min	5 min	15 min	30 min
IPP					
BP (mmHg)	119 (12.2)	117 (11.1)	119 (12.7)	122 (14.5)	123 (14.7)
	81 (6.8)	81 (7.7)	82 (6.5)	80 (7.8)	86 (6.6)
HR (/min)	123 (9.9)	124 (8.5)	123 (7.6)	126 (7.3)	130 (5.2)
CVP (cmH_2O)	10.8 (1.2)	10.4 (0.8)	11.0 (1.1)	11.6 (0.8)	11.8 (0.4)
NIPP 5%					
BP (mmHg)	116 (8.1)	116 (14.3)	118 (17.0)	127 (15.5)	121 (18.7)
	81 (11.8)	83 (17.4)	80 (8.7)	87.2 (13.7)	79 (16.1)
HR (/min)	111 (11.2)	110 (12.6)	108 (7.2)	120 (15.6)	122 (20.1)
CVP (cmH_2O)	11.2 (1.2)	10.6 (1.4)	9.6 (1.7)	10.0 (1.8)	9.8 (1.2)
NIPP 10%					
BP (mmHg)	129 (16.5)	129 (14.9)	132 (18.1)	138 (16.8)	136 (21.4)
	88 (9.5)	89 (9.7)	87 (11.7)	99 (16.2)	95 (18.9)
HR (/min)	122 (6.1)	119 (5.2)	117 (8.5)	135 (9.6)	147 (11.1)
CVP (cmH_2O)	11.8 (0.7)	11.6 (0.8)	11.2 (1.5)	11.8 (0.4)	11.6 (1.0)

BP blood pressure, *HR* heart rate, *SAT* blood oxygen saturation and *CVP* central venous pressure were measured to assess the effect of IPP or NIPP on systemic hemodynamics. Time after the start of IPP or NIPP is shown in minutes. Each value represents the results of five independent experiments
Mean (SD), $*P < 0.01$

Table 2 Platinum concentration in the NIPP circuit

	Platinum[a]		
	Group A ($n = 5$)	Group B ($n = 5$)	Group C ($n = 5$)
Plasma pelvic to systemic exposure ratio(SD) during IPP	8.9 (3.5):1.0	17.9 (10.7):1.0	75.9 (50.1):1.0
Plasma drug concentration(SD)			
Maximum pelvic concentration			
In the arterial side	54.1 (13.8)mg/l	51.0 (13.9)mg/l	55.4 (4.2)mg/l
In the venous side	45.8 (14.3)mg/l	46.6 (16.0)mg/l	45.4 (6.8)mg/l
Pelvic concentration at the end of IPP			
In the arterial side	44.9 (16.8)mg/l	43.6 (13.8)mg/l	48.8 (4.6)mg/l
In the venous side	41.1 (14.2)mg/l	40.6 (14.8)mg/l	41.0 (5.6)mg/l
Systemic venous concentration at the end of IPP	6.3 (0.7)mg/l	3.1 (0.9)mg/l	1.3 (1.1)mg/l
Average of pelvic concentration during IPP			
In the arterial side	38.2 (10.2)mg/l	35.9 (7.4)mg/l	39.1 (2.0)mg/l
In the venous side	32.7 (9.8)mg/l	32.1 (8.9)mg/l	34.2 (3.8)mg/l
Average systemic venous concentration during IPP	3.9 (0.7)mg/l	2.0 (0.6)mg/l	0.8 (0.7)mg/l
% drug (SD) eliminated in urine during IPP	5.2 (0.9)%	0.6 (0.2)%	0.5 (0.3)%
% drug (SD) eliminated in reservoir during IPP	4.2 (1.3)%	12.2 (4.7)%	21.4 (2.9)%

[a]Plasma platinum concentrations; each value is the mean of five different determinations

B, and C, and the differences between the three groups were significant ($P < 0.01$).

Discussion

Isolated pelvic perfusion is more difficult to achieve than isolated perfusion of the liver or kidney because of the highly-developed collateral vessels in the pelvis. Even though the inferior vena cava is occluded artificially or pathologically, pelvic collateral veins, such as the ascending lumbar, iliolumbar, and lateral sacral veins, contribute greatly to the return to the heart. Lawrence et al (1961, 1963) reported that the leakage of blood from the pelvic into the systemic circulation during IPP ranged from 39% to 55% as determined by radioimmunosorbent assay or with radiolabeled red cells and radiolabeled albumin. A drug leakage of 38–45% into the systemic circulation has also been reported when anticancer agents such as cisplastin or mitomycin C are administered by IPP (Collins 1989; Wile et al 1985, 1987; Turk et al 1993; Wanebo et al 1996, 1999).

When IPP is performed, the total flow-in volume in the pelvis (the blood flow through the infusion pump and the collateral vessels) becomes greater than the aspirated flow-out volume withdrawn. The systolic blood pressure allows the arterial blood flow to bypass

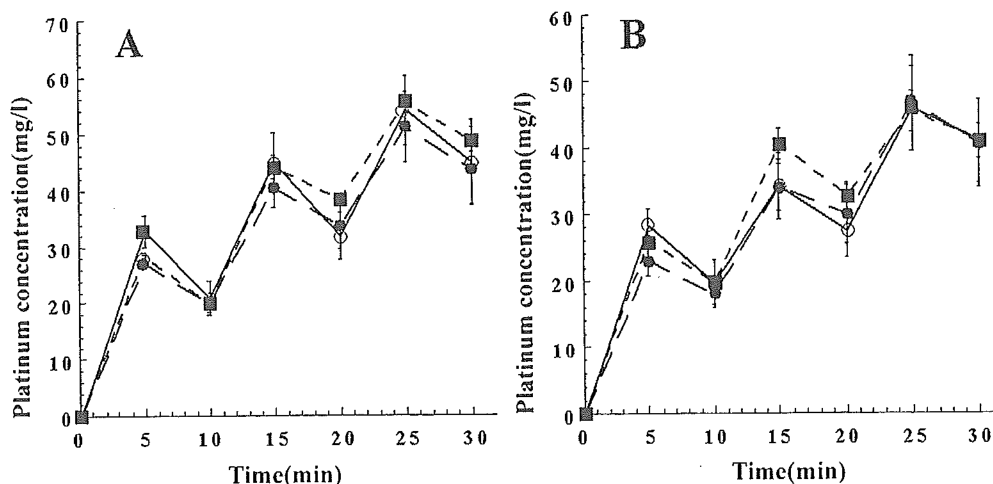

Fig. 3A–B Arterial and venous platinum concentrations in the pelvic circulation. **A** Arterial side, **B** Venous side; aspirated volume was equal to the volume returned (300 ml/min; *open circles*), 5% higher (315 ml/min; *closed circles*), and 10% higher (330 ml/min; *closed squares*)

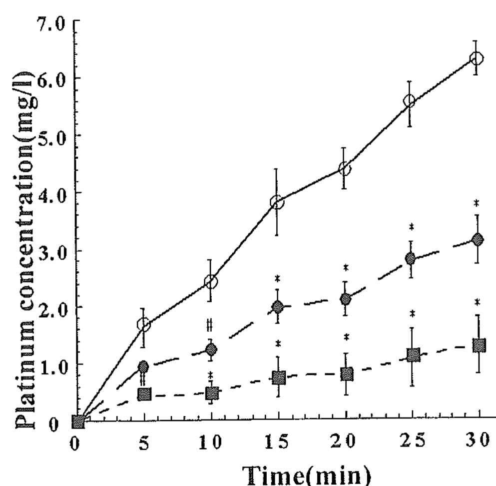

Fig. 4 Comparison of systemic venous platinum concentrations when the volume withdrawn was equal to the volume returned (300 ml/min; *open circles*), 5% higher than the volume returned (315 ml/min; *closed circles*), and 10% higher than the volume returned (330 ml/min; *closed squares*)

the aortic occlusion through the arterial collaterals, leading to a larger pelvic blood volume, which then results in a higher venous pressure in the pelvic circulation than in the systemic circulation. This pressure gradient is thought to be the cause of the leakage of anticancer agents into the systemic circulation through the pelvic venous collaterals. A low-pressure pump system with infusion rates of 100–200 ml/min has been used in the past to reduce such leakage (Turk et al 1993; Wanebo et al 1996, 1999), but this was not shown to be effective.

To solve this problem, we devised a new system, NIPP, in which twin rotary pumps independently adjust the volume of perfused blood withdrawn and returned. In this study, we used this new instrumentation to investigate the effect of changing the volume of blood withdrawn during IPP on leakage of the anticancer drug nedaplatin into the systemic circulation, and our data demonstrated that increases in volume withdrawn significantly decreased drug leakage and that the concentration of the anticancer drug in the pelvic circulation remained high.

The drug concentration in the systemic circulation in group C, in which 10% more blood was withdrawn, was significantly lower than in group B, where 5% more was withdrawn. The difference in urinary excretion of the drug between groups B and C was not significant: less than 1% of the infused drug was excreted in the urine in both groups, whereas 5% was excreted in group A. Thus, the 5% higher withdrawal in IPP seems to be sufficient to reduce the distribution of nedaplatin to the kidney, although 10% higher withdrawal may be more favorable when the patient has a serious condition such as renal failure. However, it should be noted that reduction of blood volume by increasing the volume withdrawn would cause anemia in patients and would require auto-transfusion and/or an intravenous drip infusion during NIPP therapy.

In summary, withdrawal of a greater blood volume by IPP using a twin-pump system with a negative-balance in-out flow, which we refer to as NIPP, will overcome the major drawback of conventional IPP therapy in clinical situations. Therefore, we suggest that NIPP may improve the practicability of IPP, and it will aid patients with inoperable and advanced pelvic malignancies which tend to renal dysfunction due to hydronephrosis. The merit of the NIPP system is that it simply requires the addition of twin rotary pumps to the existing IPP system in order to control the in-out flow rate. However, a problem remains in that the concentration of the drug in the pelvic circulation still needs to be decreased after NIPP therapy. We suggest that this problem might be resolved by using isolated dialysis of anticancer agents in the pelvic cavity to decrease the concentration of the drug after NIPP therapy. Based on the above findings, we have performed NIPP therapy in patients with advanced, inoperable or recurrent cancer, and obtained good control of tumor progression without complications.

In conclusion, the NIPP system we have developed may pave the way to improved IPP therapy that will

reduce the side effects of anticancer drugs without compromising their therapeutic efficacy in the pelvic circulation.

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Adenovirus-Mediated Overexpression of REIC/Dkk-3 Selectively Induces Apoptosis in Human Prostate Cancer Cells through Activation of c-Jun-NH_2-Kinase

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Abstract

Alteration in genes which takes place during malignant conversion and progression could be potential targets for gene therapy. We previously identified *REIC/Dkk-3* as a gene whose expression is reduced in many human cancers. Here, we showed that expression of REIC/Dkk-3 was consistently reduced in human prostate cancer tissues in a stage-dependent manner. Forced expression of REIC/Dkk-3 induced apoptosis in human prostate cancer cell lines lacking endogenous REIC/Dkk-3 expression but not in REIC/Dkk-3-proficient normal prostate epithelial and stromal cells. The apoptosis involved c-Jun-NH_2-kinase activation, mitochondrial translocation of Bax, and reduction of Bcl-2. A single injection of an adenovirus vector carrying REIC/Dkk-3 showed a dramatic antitumor effect on a xenotransplanted human prostate cancer. Thus, REIC/Dkk-3 could be a novel target for gene-based therapy of prostate cancer. (Cancer Res 2005; 65(21): 9617-22)

Introduction

Selective elimination of tumor cells is a key issue in treating human cancer. During malignant conversion and progression, various genetic changes take place in cells that could be potential targets of cancer gene therapy. Among the genes that exert a selective killing effect on cancer cells when overexpressed are p53 (1, 2) and mda-7/interleukin-24 (mda-7/IL-24; ref. 3). The Dickkopf (*Dkk*) gene family is known to interfere with Wnt signaling via Wnt-receptors (4, 5). Wnt genes play pleiotropic roles in critical biological contexts including development, cell growth/differentiation, and cancer (6). Although Dkk family members (four genes are known thus far in humans) are only vaguely understood, they probably fulfill important functions as well. We previously identified *REIC/Dkk-3* as a gene whose expression is reduced in many human cancers (7–10). Forced expression of REIC/Dkk-3 using a plasmid vector inhibited growth of a human osteosarcoma cell line (8). These findings indicate that REIC/Dkk-3 may function as a tumor suppressor gene and could provide a new means of treatment for some types of human cancer. In this study, we showed that overexpression of REIC/Dkk-3 selectively induced apoptotic cell death in human prostate cancer cells via activation of c-Jun-NH_2-kinase (JNK) and exerted a marked curing effect on xenotransplanted human prostate cancer.

Materials and Methods

Reagents, cells, and culture. Normal human prostate epithelial cells (PrEC) and prostate stromal cells (PrSC) cells were purchased from Cambrex (Baltimore, MD) and cultivated using medium recommended by the supplier. The prostate cancer cell lines PC3, DU145, and LNCaP were provided by American Type Culture Collection (Rockville, MD). Normal human fibroblasts (OUMS-24) were established by one of the authors (11). HAM'S F-12 K medium, RPMI 1640, and DMEM (Nissui, Tokyo, Japan) were used for PC3, DU145 and LNCaP, and OUMS-24, respectively, with a supplement of 10% fetal bovine serum. Subfractionation of cells was done using a Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA). A JNK inhibitor, SP600125, and a membrane-permeable Bax inhibitor, V5, were purchased from Biomol (Plymouth Meeting, PA) and Sigma Genosys (Woodlands, TX), respectively.

Human prostate tissues. LandMark low-density prostate tissue microarray (Ambion, Austin, TX) was used for immunostaining of REIC/Dkk-3. Fresh prostate biopsy samples were obtained from 40 patients under conditions permitted by the Ethical Board of our Graduate School. Twenty samples each had Gleason scores of ≤7 and scores of 8 to 10, respectively.

Immunologic analyses. After fixation with 4% paraformaldehyde, cells and tissue sections were immunostained with anti-human REIC/Dkk-3 antibody raised in our laboratory, followed by treatment with Alexa fluor R594-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). For Bax and Bcl-2, rabbit anti-human Bax antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) and mouse anti-human Bcl-2 antibody (BD Biosciences, San Diego, CA) were used, respectively. Vectashield mounting medium with 4′,6-diamidino-2-phenylindole or with propidium iodide (Vector Laboratories, Burlingame, CA) was used for counterstaining of cell nuclei. SyBR Green I (Cambrex) was used for nuclear staining in tissue sections. Signal intensity of the stained samples was quantitated using the computer software Scion Image Beta (Scion, Frederick, ML). Western blot analysis was done as previously described. The antibodies used were as follows: rabbit anti-human REIC/Dkk-3 antibodies raised in our laboratory for REIC/Dkk-3; Apoptosis Sampler I kit (BD Biosciences) for Bcl-2 and Bcl-xL; rabbit anti-human Bax antibody (Upstate Cell Signaling Solutions); mouse anti-human tubulin antibody (Sigma, St. Louis, MO); mouse anti-horse cytochrome *c* antibody (Upstate Biotechnology, Lake Placid, NY); mouse anti-mouse mitochondrial Hsp70 (Abcam, Cambridge, United Kingdom); rabbit anti-human c-Jun antibody, rabbit anti-human phospho-c-Jun (Ser^{63}) antibody, rabbit anti-human stress-activated protein kinase/JNK antibody, and rabbit anti-human phospho–stress-activated protein kinase/JNK (Thr^{183}/Tyr^{185}) antibody (Cell Signaling Technology, Beverly, MA); mouse anti-β-galactosidase antibody (Calbiochem, San Diego, CA).

Real-time quantitative reverse transcription-PCR. Real-time PCR was done under the conditions recommended by the manufacturer using a LightCycler rapid thermal cycler instrument (Roche Diagnostic, Lewes, United Kingdom). The primers used for real-time PCR were as follows:

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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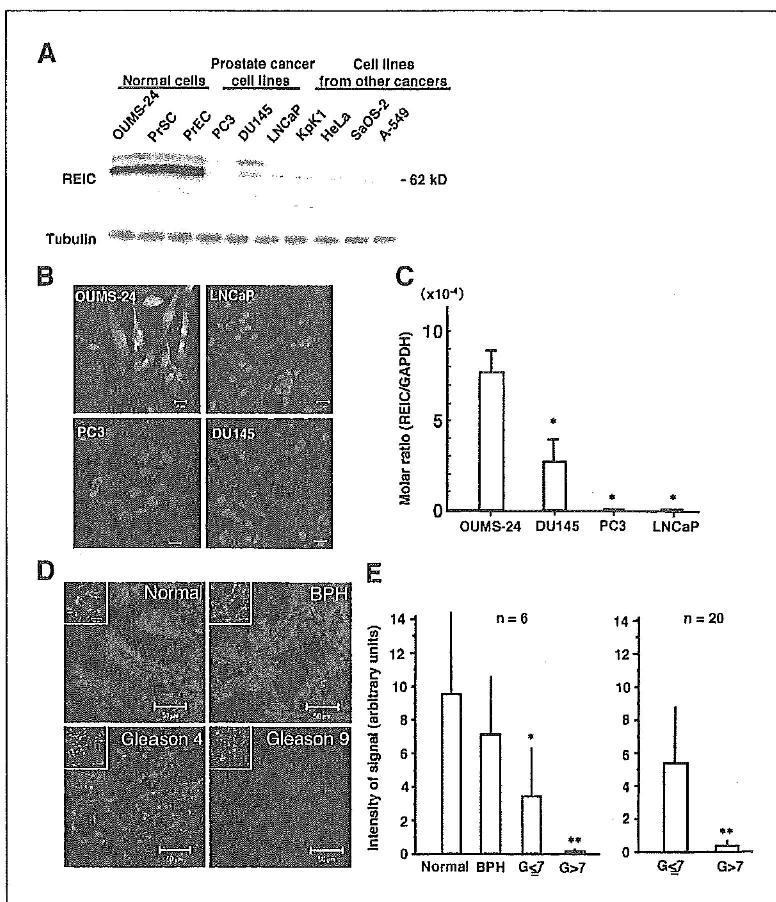

Figure 1. Reduced expression of REIC/Dkk-3 in human prostate cancer cells and tissues. *A*, REIC protein level was determined by Western blot analysis in normal human cells (OUMS-24, normal human fibroblasts; PrSC, prostate stromal cells; and PrEC, prostate epithelial cells) and human cancer–derived cell lines (PC3, DU145, and LNCaP, prostate cancer; KpK-1, renal cancer; HeLa, cervical cancer; SaOS-2, osteosarcoma; A-549, lung cancer). Tubulin was used as an internal control. *B*, immunocytochemistry for REIC/Dkk-3 protein (*green*) in OUMS-24 and prostate cancer cell lines, PC3, DU145, and LNCaP. The cells were counterstained with propidium iodide for nuclear DNA (*red*). *C*, REIC/Dkk-3 mRNA level of normal human fibroblasts and prostate cancer cell lines was determined by real-time quantitative RT-PCR and expressed as molar ratios to those of GAPDH. *, $P < 0.05$ compared with OUMS-24. *D*, immunohistochemistry for REIC/Dkk-3 in normal and benign prostate hyperplasia (*BPH*) tissues and prostate cancer tissues at Gleason stages 4 and 9. *Insets*, low-magnification tissue staining with SyBR Green. *E*, quantitative image analysis for REIC/Dkk-3 protein in LandMark tissue microarray (*left*, 6 cases each) and in freshly isolated human prostate cancer tissues (*right*, 20 cases each). The intensity of signals was quantitated using the computer software, Scion Image Beta. *BPH*, benign hypertrophic prostate tissues; *G*, Gleason stages; *, $P < 0.05$; **, $P < 0.01$.

REIC/DKK-3 (forward) 5′-GTAAGTTCCCCTCTGGCTTG-3′, REIC/Dkk-3 (reverse) 5′-AAGCACCAGACTGTGAAGCCT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward) 5′-GGGTGTGAACCATGAGAAGTATGA-3′, GAPDH (reverse) 5′-TGCTAAGCAGTTGGTGGTGC-3′. The products were checked by a melting point analysis, electrophoretic mobility, and direct sequencing. Standard curves for calculation of the number of transcripts were made using plasmid containing the respective inserts. The results are shown as molar ratios of REIC/Dkk-3 to GAPDH transcripts.

Adenovirus vector carrying REIC/Dkk-3. For overexpression of REIC/Dkk-3, a full-length cDNA was integrated into a cosmid vector pAxCAwt and transferred into an adenovirus vector by the COS-TPC method (Takara Bio, Shiga, Japan). An adenovirus vector carrying *LacZ* gene was used for monitoring infection efficiency.

Apoptosis assay. Detection of DNA ladders was done under conventional conditions. Briefly, DNA was gently extracted after lysing cells with Triton X-100, treated with RNase and proteinase K, and electrophoresed on 2% agarose gel. *In situ* Cell Death Detection Kit, Fluorescein (Roche, Penzberg, Germany) was used for terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay. Mitotracker Orange CMTMRos was purchased from Molecular Probes.

***In vivo* experiments.** PC3 cells (2.5×10^6 in 50 μL PBS) were mixed with 50 μL Matrigel (BD Biosciences) and s.c. injected into the right flank of 8-week-old BALB/C nude mice (SLC, Hamamatsu, Japan). One week after injection, when the tumor diameter reached ~5 mm, 2.5×10^8 plaque-forming units of an adenovirus vector carrying full-length REIC/Dkk-3 cDNA (Ad-REIC) or LacZ (Ad-LacZ) in a 100 μL buffer were injected intratumorally. The same volume of buffer was injected as a negative control. The size of tumors was measured every 3 days over 30 days after the injection. Tumor volume was calculated using an empirical formula, $V = 1/2 \times$ [(the shortest diameter)^2 × (the longest diameter)]. The experiments were done according to a guideline determined in our university.

Results and Discussion

Reduction of REIC/Dkk-3 expression in human prostate cancer cells and tissues. We first examined its expression in various cell lines. In normal human fibroblasts (OUMS-24), and prostate epithelial (PrEC) and stromal (PrSC) cells, REIC/Dkk-3 protein was detected as two major bands of ~60 and ~68 kDa in size (Fig. 1*A*). REIC/Dkk-3 gave rise to several bands of different apparent molecular size, probably due to variable glycosylation levels (10). REIC/Dkk-3 protein was barely detected in three prostate cancer cell lines and four cancer cell lines of other origin. Lack of expression of REIC/Dkk-3 protein in the prostate cancer cell lines was confirmed by immunostaining (Fig. 1*B*). Quantitative RT-PCR also revealed a reduction in REIC/Dkk-3 mRNA levels in the prostate cancer cell lines (Fig. 1*C*).

Expression of REIC/Dkk-3 was examined in human prostate tissues by immunostaining (Fig. 1*D* and *E*). REIC/Dkk-3 was detected in epithelial and stromal cells in normal and benign hypertrophic prostates. In both commercially available tissue microarray (Fig. 1*E, left*) and freshly obtained prostate cancer tissues (Fig. 1*E, right*), REIC/Dkk-3 was reduced in cancer cells depending on the grade of malignancy. Prostate cancer tissues with Gleason scores of 8 to 10 were consistently negative in the expression of REIC/Dkk-3. Immunostaining of the tissue microarray also revealed that all the normal human tissues examined, including brain, heart, liver, pancreas, kidney, mammary gland, and lymph node, were positive for REIC/Dkk-3 expression, with varying intensities depending on the cell type (data not shown).

These results extend those that we have obtained previously by Northern blot analysis (7).

Selective induction of apoptosis in human prostate cancer cell lines by overexpression of REIC/Dkk-3. To examine the possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy, we overexpressed REIC/Dkk-3 using a replication-deficient adenovirus vector. The REIC/Dkk-3 protein level in PC3 cells infected with the vector carrying REIC/Dkk-3 (Ad-REIC) at a multiplicity of infection (MOI) of 1 was comparable to the endogenous REIC/Dkk-3 level of OUMS-24 (Fig. 2*A*). Within a few days after infection, most of the prostate cancer cells, but not normal cells, were detached from the bottom of culture vessels. The infection efficiency of an adenovirus vector (~12% at the MOI of 0.1 and ~95% at the MOI of 1.0; Fig. 2*B*) and the expression level of REIC/Dkk-3 after infection with Ad-REIC (Fig. 2*C*) were similar between OUMS-24 and PC3 cells. To explore the cause of the detachment, we stained the cells by the TUNEL method 36 hours after the infection. As shown in Fig. 2*D*, many TUNEL-positive cells were observed in prostate cancer cell lines (PC3, DU145, and LNCaP) but not among normal cells (OUMS-24, PrEC, and PrSC). The incidence of TUNEL-positive cells was 49%, 24%, and 41% in PC3, DU145 and LNCaP, respectively, whereas <1% of cells were TUNEL-positive among normal cells (Fig. 2*E*). A clear DNA ladder was observed in the samples prepared from PC3 cells 36 hours after the infection with Ad-REIC at MOIs >1 (Fig. 2*F*). These results indicate that overexpression of REIC/Dkk-3 selectively induces apoptotic cell death in prostate cancer cell lines that, for the most part, lack endogenous REIC/Dkk-3 expression.

Mechanistic insights into the cancer cell–specific induction of apoptosis by REIC/Dkk-3. We screened the expression levels of various apoptosis/cell cycle regulation–related proteins in PC3, the most sensitive cell line among those examined, and compared them with those in OUMS-24. Infection of PC3 cells with Ad-REIC reduced the level of antiapoptotic Bcl-2 and Bcl-xL proteins (Supplemental information; Fig. 3*F*). No significant change in the levels of Bax, Bad, Apaf-1, p53, p21 (CIP1/WAF1), or p16 (INK4a) was observed in either cell type. Although a caspase-8 inhibitor did not block Ad-REIC-induced apoptosis in PC3 cells (data not shown), a membrane-permeable Bax inhibitor V5 completely abrogated the apoptosis (Fig. 3*A* and *B*). Translocation of Bax protein from the cytoplasm to the mitochondria is a hallmark of triggering a Bax-mediated apoptotic pathway. Figure 3*C* shows that Bax protein was translocated to the mitochondria by Ad-REIC and the translocation was suppressed by V5. V5, in itself, showed no effect on the protein levels of Bcl-2 and Bax (data not shown).

One of the upstream activators of Bax is a JNK (12, 13), which promotes translocation of Bax to mitochondria (13). When we applied a JNK-specific inhibitor, SP600125, to PC3 cells, the Ad-REIC-induced apoptosis of PC3 cells was remarkably abrogated in a dose-dependent manner (Fig. 3*D* and *E*). Activation of JNK in PC3 cells infected with Ad-REIC was confirmed using a phosphorylated JNK-specific antibody and by detecting the phosphorylation of c-Jun (Fig. 3*F*). SP600125 is known to inhibit the kinase activity of JNK but not the phosphorylation of JNK itself. Bax protein was detected in the cytoplasm of growing PC3 cells but translocated to mitochondria in the cells infected with Ad-REIC (Fig. 3*G*). The mitochondrial translocation of Bax protein was associated with the release of cytochrome *c* into the cytoplasm and was suppressed by SP600125. JNK was partly translocated to mitochondria by Ad-REIC. These results indicate that overexpression of REIC activates JNK, reduces the Bcl-2 protein level, induces mitochondrial translocation of Bax protein, releases cytochrome *c* into the cytoplasm, and finally leads to apoptotic cell death. Recently, Hsieh et al. (14) reported that overexpression

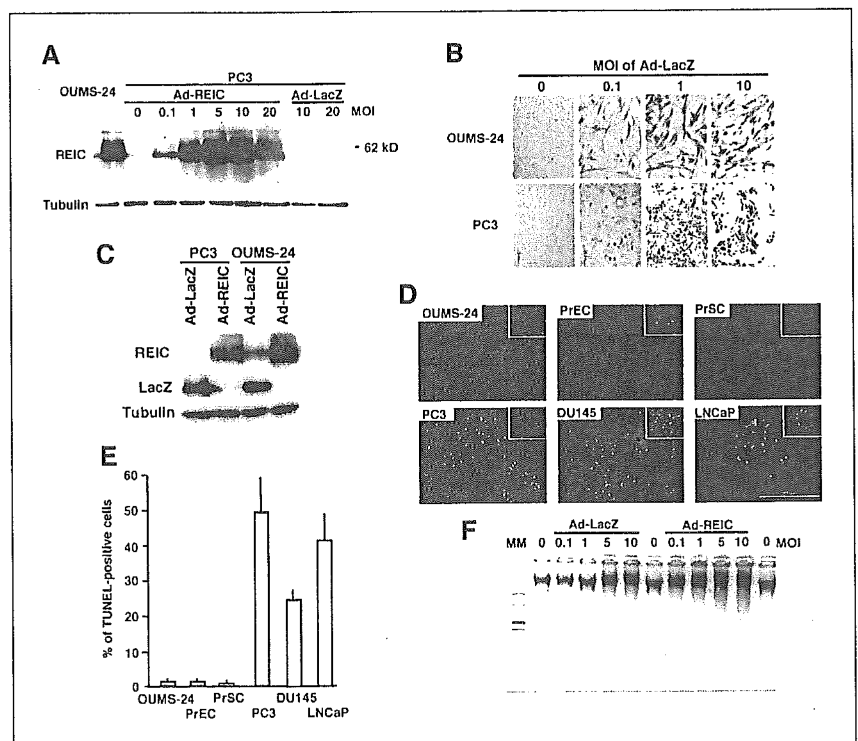

Figure 2. Specific induction of apoptosis in human prostate cancer cell lines by overexpression of REIC/Dkk-3. *A*, expression of REIC/Dkk-3 protein in a human prostate cancer cell line PC3 36 hours after infection with an adenovirus vector carrying full-length REIC/Dkk-3 cDNA (Ad-REIC) at different MOIs. Uninfected OUMS-24 was used as a positive control. Ad-LacZ, an adenovirus vector carrying LacZ. *B*, infection efficiency of Ad-LacZ to OUMS-24 and PC3 cells as visualized by staining with X-gal. *C*, expression of REIC/Dkk-3 in OUMS-24 and PC3 cells after infection with Ad-REIC at 10 MOI. *D*, TUNEL staining (*green*) of normal human cells (OUMS-24, PrEC, and PrSC) and prostate cancer cell lines (PC3, DU145, and LNCaP) fixed 36 hours after the infection at 10 MOI. *Insets*, cells stained with 4′,6-diamidino-2-phenylindole (*blue*). *E*, percentage of TUNEL-positive cells counted under the same conditions as those in (*D*). *F*, characteristic fragmentation of DNA was observed in PC3 cells infected with Ad-REIC at MOIs >1. One day after plating of 5 × 10^5 PC3 cells, the cells were infected with the indicated virus vectors at various MOI and harvested 36 hours later.

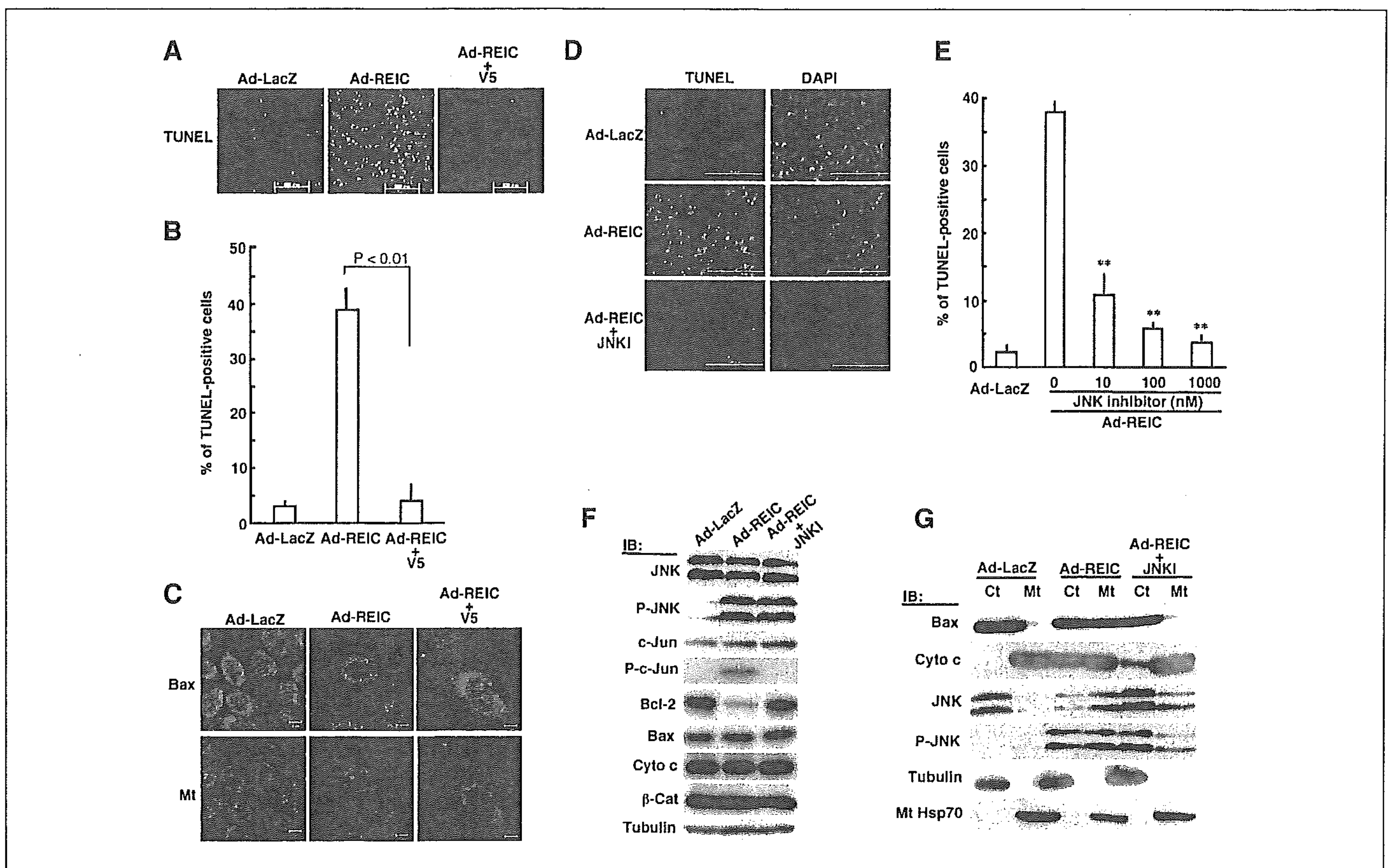

Figure 3. Involvement of Bax and JNK in the induction of apoptosis in PC3 cells by Ad-REIC. *A,* TUNEL staining of PC3 cells 36 hours after infection with an adenovirus vector carrying LacZ (Ad-LacZ) or REIC/Dkk-3 (Ad-REIC) at 10 MOI. V5 was added at 200 μmol/L into the medium 1 hour prior to the infection. *Bars,* 200 μm. *B,* suppression of REIC/Dkk-3-induced apoptosis in PC3 cells by a Bax-inhibitor V5. Number of TUNEL-positive cells was counted under the same conditions as those in (*A*). *C,* immunocytochemistry for Bax protein in PC3 cells 36 hours after infection of the virus vectors at 10 MOI. Intracellular localization of mitochondria was visualized using Mitotracker (*Mt*). *D,* TUNEL staining of PC3 cells under the same conditions with those used in (*A*) except for the use of JNK inhibitor SP600125 at 10 nmol/L. *E,* suppression of REIC/Dkk-3-induced apoptosis in PC3 cells by a JNK inhibitor SP600125. The number of TUNEL-positive cells was counted under the same conditions as those in (*C*). *F,* Western blot analysis for the indicated proteins in PC3 cells treated similarly with those in (*D*). *P-,* phosphorylated; *Cyto c,* cytochrome *c*; *β-Cat,* β-catenin. *G,* Western blot analysis for indicated proteins in cytoplasm (*Ct*) and mitochondria (*Mt*) prepared from PC3 cells treated similarly with those in (*D*).

of REIC/Dkk-3 induced apoptotic cell death in several types of human cancer cell through activation of caspase-3, which is known to be a major apoptosis executor in the down-streaming of cytochrome *c*.

At present, it is not clear whether JNK activated by Ad-REIC acts on Bcl-2 and Bax directly or indirectly, although Bcl-2 (15), Bcl-xL (16), and Bim (17) have been identified as targets of both JNK and c-Jun. We confirmed that the REIC/Dkk-3 protein was glycosylated and secreted into the culture medium when overexpressed in PC3 cells. The amount and intracellular localization of β-catenin were not affected by Ad-REIC (Fig. 3*F*; data not shown), excluding the possibility of involvement of the canonical pathway of Wnt signaling. JNK may be activated through the noncanonical pathway of Wnt signaling by secreted REIC/Dkk-3; more likely, it is activated intracellularly through a stress-sensing system for the overexpressed protein or through yet unknown intracellular target proteins of REIC/Dkk-3. In this context, it should be noted that mda-7/IL-24, which also selectively induces apoptosis in many different types of human cancer cells, efficiently induced apoptosis in human prostate cancer cell lines even when the secretion was blocked by truncating the signal peptide (17). Although Bax is activated by p53 under certain conditions (18), induction of apoptosis by Ad-REIC is apparently independent of p53 function because PC3 is null in p53 (Supplemental information; ref. 19). This is true also of mda-7/IL-24, which exerts its effect through p53-independent activation of Bax (20). Recently, Hoang et al. (5) reported that overexpression of REIC/Dkk-3 did not induce apoptosis in a human osteosarcoma cell line, Saos-2, but inhibited invasion and motility of the cells *in vitro*.[4] REIC/Dkk-3 may exert its anticancer activity at different action points.

REIC/Dkk-3-targeted gene therapy for prostate cancer in a xenotransplantation model. Because selective growth inhibition and apoptosis by overexpression of REIC/Dkk-3 were indicated by *in vitro* studies, we investigated the effect of Ad-REIC on the growth of PC3 cells *in vivo*. PC3 cells of 2.5×10^6 in Matrigel were transplanted s.c. into nude mice. One week after the transplantation, when the tumor volume reached 30 to 100 mm^3, 2.5×10^8 plaque-forming units of Ad-REIC or Ad-LacZ in 100 μL were injected intratumorally. The tumors progressively grew in the buffer-injected control and Ad-LacZ-injected groups during

[4] In accord with our unpublished data which was obtained under similar conditions.

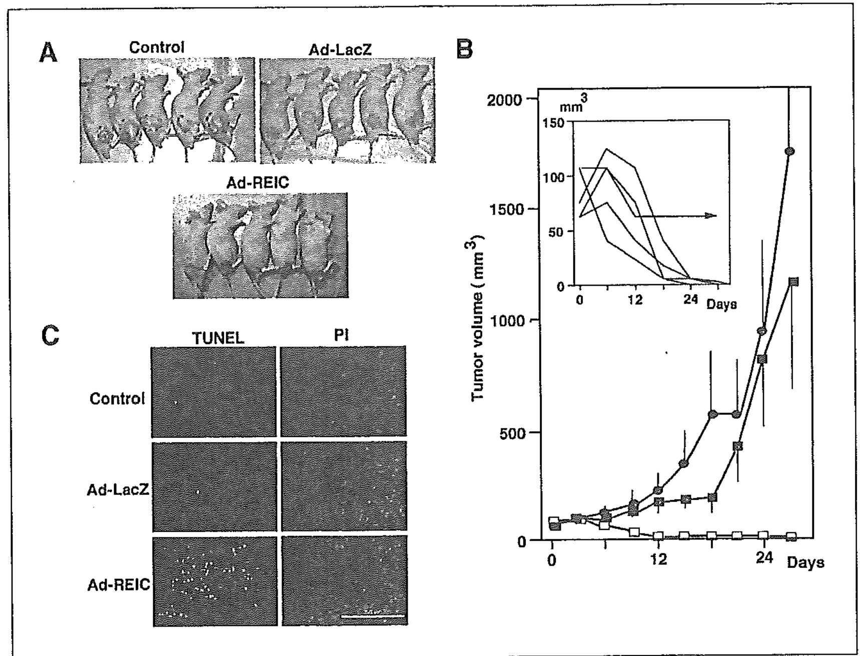

Figure 4. Effect of Ad-REIC on the growth of PC3 in nude mice. *A*, appearance of tumors at the end of the observation period. *B*, mean volume of tumors estimated from the diameters in five nude mice in each group. ●, PBS; ■, Ad-LacZ; □, Ad-REIC; *vertical bars*, SD. *Insets*, volume of each tumor in the nude mice injected with Ad-REIC. Four of the five mice were completely tumor-free when autopsied 30 days after the virus injection. *C*, TUNEL staining of tumor tissues obtained on autopsy 30 days after the virus injection. *PI*, stained with propidium iodide to visualize nuclei.

the observation period of 1 month (Fig. 4*A* and *B*). In contrast, the tumors completely disappeared in four out of five mice in the group receiving Ad-REIC injection; even in the tumor-bearing mouse in this group, the tumor did not actively grow and remained unchanged throughout the observation period. An ~2-fold increase in tumor size was observed in two of the five transplanted tumors during the first week, possibly due to the lag time until the availability of fully functional REIC/Dkk-3 protein and local effects of injection, including edema and inflammation. The tumors were resected at the end of observation and examined by TUNEL staining (Fig. 4*C*). No apoptotic cells were observed in tumors injected with the buffer or with Ad-LacZ, whereas many cells were positive in TUNEL staining even 1 month after the injection of Ad-REIC in the residual tumor.

The sharply selective induction of apoptosis in culture that was observed and the highly efficient inhibition of tumor growth *in vivo* by overexpression of REIC/Dkk-3 imply extraordinarily promising characteristics of REIC/Dkk-3 as a target gene for cancer therapy, possibly comparable to p53 and mda-7/IL-24 (17). Prostate cancer is the most commonly diagnosed malignancy in many Western countries. Various therapeutic measures including anti-androgen therapy have been applied to prostate cancer with considerable success. However, once prostate cancers acquire androgen-independent growth capabilities at later stages, as did PC3, they are hardly controlled by the conventional therapies and often exhibit lethality. It is hoped that our present results lead to the identification of a new molecular target for counteracting this notoriously vicious disease.

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