Virchows Arch (1996) 428:325–331

ORIGINAL ARTICLE

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Immunohistochemical study of arginase in cancer of the stomach

Received: 7 March 1996 / Accepted: 15 April 1996

Abstract High levels of arginase have been detected in gastric adenocarcinoma. To examine the hypothesis that this is due to macrophage infiltration into the tumour, we localized the cellular distribution of arginase by immunohistochemical staining. We examined gastric adenocarcinomas and their corresponding normal tissues (n = 45), leiomyomas (n = 2), leiomyosarcomas (n = 3), human gastric adenocarcinoma cell lines (n = 3), and benign gastric ulcers (n = 4) by the avidin–biotin–peroxidase complex technique. Macrophages with strong arginase immunoreactivity were observed infiltrating both gastric normal and cancer tissues. No arginase immunoreactivity was observed in normal mucosal gland, muscular and serosal tissues or benign gastric ulcers. The immunoreactivity of arginase was positive but heterogeneous in most specimens of gastric adenocarcinoma (62.2%) and was absent from gastric intestinal metaplasia, leiomyomas and leiomyosarcomas. Among the 28 neoplasms with arginase immunoreactivity, scattered immunoreactivity was also noted in adjacent dysplastic glands in 12 (42.8%) specimens. Arginase immunoreactivity was observed in all three gastric cancer cell lines. Arginase is present in the cytoplasm but not in the nucleus. These data suggest that the high arginase levels in adenocarcinoma cancer tissues originate largely from cancer cells.

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Key words Arginase · Gastric cancer · Immunohistochemistry

Introduction

The main function of human hepatic arginase is the production of urea in the detoxification of ammonia [3], but the metabolic function of extrahepatic arginase in erythrocytes [2], leucocytes [3], macrophages [9] and kidney [3] remains unclear. Moreover, many studies [6, 15, 17] also indicate a potent immune inhibitory effect of arginase.

Several reports, including ours, have shown that high arginase levels exist in human carcinomas, including prostatic [4], colon [11] and gastric cancers [20, 21]. We also found that gastric cancer patients had elevated serum arginase levels [23], which might suppress the patients' natural and activated splenic killer cell activities [24]. In addition, the production of arginase in gastric cancer cell lines is regulated by glucocorticoid [22].

The purpose of this study was to localize the cellular distribution of arginase histochemically in normal and cancer gastric tissues. In particular, we were interested in examining the hypothesis that the high levels of arginase in malignant tissues are due to macrophage infiltration into the tumour and do not originate from cancer cells [14].

Materials and methods

Surgical specimens

Human gastric tissues were obtained at surgery and included gastric adenocarcinoma with corresponding normal gastric tissues (n = 45), leiomyoma (n = 2), and leiomyosarcoma (n = 3). We selected these cases confirmed to be of smooth muscle origin by immunocytochemical studies showing them to be weakly to strongly positive for the HHF35 smooth muscle marker. Tumours diagnosed as stromal cell tumours were not included in this study.

Gastric adenocarcinomas are divided into intestinal and diffuse by Lauren's histological criteria [10], and they exist either alone or

Table 1 Status of arginase staining and clinicopathological features in patients with gastric cancer (– no positive cells, + 1–25%, ++ 26–75%, +++ more than 75% in non-overlapping microscopic fields, *A* antrum, *M* midbody, *C* cardia, *D* duodenum, *E* oesophagus, *Mod* moderately differentiated, *Poor* poorly differentiated, *Well* well differentiated, α lesion showing expansive growth and distinct border from surrounding tissue, γ lesion showing infiltrative growth with ill-defined border, β intermediate growth pattern between α and β)

No.	Arginase immuno-reactivity	Sex	Age (year)	Gross features			Microscopical features				Clinical stage
				Tumour location	Size (cm)	Borrmann type	Grading	Intestinal type:	Diffuse type	Growth pattern	
1	+++	Male	75	Am	7	3	Poor	95%:	5%	γ	IV
2	+++	Male	68	Mac	15	4	Poor	5%:	95%	γ	IV
3	+++	Male	64	Mac	8.5	4	Poor	5%:	95%	γ	III
4	+++	Female	42	M	4.5	3	Poor	0:	100%	γ	II
5	++	Male	80	Ma	7	3	Poor	100%:	0	γ	III
6	++	Female	65	Ad	6	3	Well	100%:	0	γ	III
7	++	Female	79	A	9	2	Mod	95%:	5%	γ	III
8	++	Male	69	A	4.5	3	Mod	90%:	10%	β	II
9	++	Female	68	A	6.5	3	Poor	50%:	50%	β	I
10	++	Male	84	A	7.8	3	Poor	40%:	60%	γ	III
11	++	Male	53	Am	7.2	2	Poor	30%:	70%	β	II
12	++	Male	67	Ma	3.9	3	Poor	25%:	75%	γ	IV
13	++	Male	80	Ma	7	3	Poor	20%:	80%	γ	III
14	++	Male	69	Mc	9.5	2	Poor	10%:	90%	β	III
15	++	Male	72	Am	7.2	3	Poor	10%:	90%	β	II
16	++	Female	42	Ma	4.5	3	Poor	5%:	95%	γ	IV
17	+	Male	69	A	6	3	Mod	100%:	0	α	III
18	+	Male	68	Ad	6.5	3	Mod	100%:	0	γ	II
19	+	Male	75	Ad	5.8	3	Mod	100%:	0	β	IV
20	+	Male	65	A	2.2	2	Mod	100%:	0	γ	II
21	+	Male	69	M	4.8	2	Mod	90%:	10%	γ	III
22	+	Male	53	A	3.1	3	Well	70%:	30%	γ	III
23	+	Male	72	Am	6.6	2	Poor	70%:	30%	β	I
24	+	Male	72	Am	6.3	4	Poor	50%:	50%	γ	III
25	+	Male	69	M	6.5	3	Poor	50%:	50%	γ	IV
26	+	Male	71	Am	7	3	Poor	40%:	60%	γ	II
27	+	Male	71	Cm	10	4	Poor	40%:	60%	γ	IV
28	+	Male	78	C	4.2	3	Poor	5%:	95%	β	III
29	–	Female	37	Ce	3.5	3	Poor	100%:	0	γ	III
30	–	Male	35	Am	7.5	3	Poor	100%:	0	γ	IV
31	–	Male	80	Ad	6.5	0	Poor	100%:	0	β	IV
32	–	Male	67	A	7.3	0	Poor	100%:	0	γ	II
33	–	Male	74	A	3.5	3	Mod	100%:	0	β	I
34	–	Male	70	M	5.5	0	Poor	90%:	10%	β	I
35	–	Male	63	A	3.8	3	Mod	90%:	10%	β	II
36	–	Male	71	A	5	3	Poor	80%:	20%	γ	III
37	–	Male	71	Ma	6.5	3	Well	70%:	30%	γ	II
38	–	Male	68	Mc	5.7	3	Mod	70%:	30%	β	IV
39	–	Male	56	A	9	3	Poor	20%:	80%	γ	III
40	–	Male	72	Ma	7.5	3	Poor	10%:	90%	β	IV
41	–	Female	55	C	5.5	3	Poor	5%:	95%	γ	IV
42	–	Male	70	Amc	16	4	Poor	0:	100%	γ	III
43	–	Female	53	Am	7	2	Poor	0:	100%	γ	II
44	–	Male	45	Am	4	3	Poor	0:	100%	γ	IV
45	–	Female	62	Amc	13	4	Poor	0:	100%	γ	IV

in combination in gastric carcinomas. Their clinicopathological features are summarized in Table 1. Cytological similarity to the normal glandular cells was graded according to the WHO International Histological Classification [19], and growth patterns were classified according to the Japanese system used in stomach cancer studies [7]. Four specimens with benign gastric ulcers located at the angularis were included. The mean age of the four patients (3 male and 1 female) was 72 years. Informed consent was obtained from all patients. The maximum time between stomach removal and quenching of the tissue was 1 h. Tissue blocks were fixed overnight at 4°C with 4% neutral buffered paraformaldehyde, dehydrated, cleared with Hemo-De (Fisherbrand, ingredients: *d*-limonene, butylated hydroxanisole), then embedded in wax. Sections 5 μm thick were used for staining.

Tumour cell lines

Three human gastric cancer cell lines were used: KATO-III [16], AGS [1] and SC-MI [8]. KATO-III cells were cultured from a metastasis in the pleural effusion of a signet-ring cell carcinoma. AGS and SC-M1 were cultured from primary gastric cancer tissue. KATO-III and SC-M1 cells were cultured in RPMI-1640 and AGS cells, in Ham's F12 medium. The culture media contained 10% fetal calf serum, kanamycin 100 μg/ml and amphotericin 1 μg/ml. For each of three human gastric cancer cell lines, 1×10^8 cells were harvested by trypsin-EDTA, then spun down and fixed in 4% neutral buffered paraformaldehyde overnight before being embedded in wax as described above.

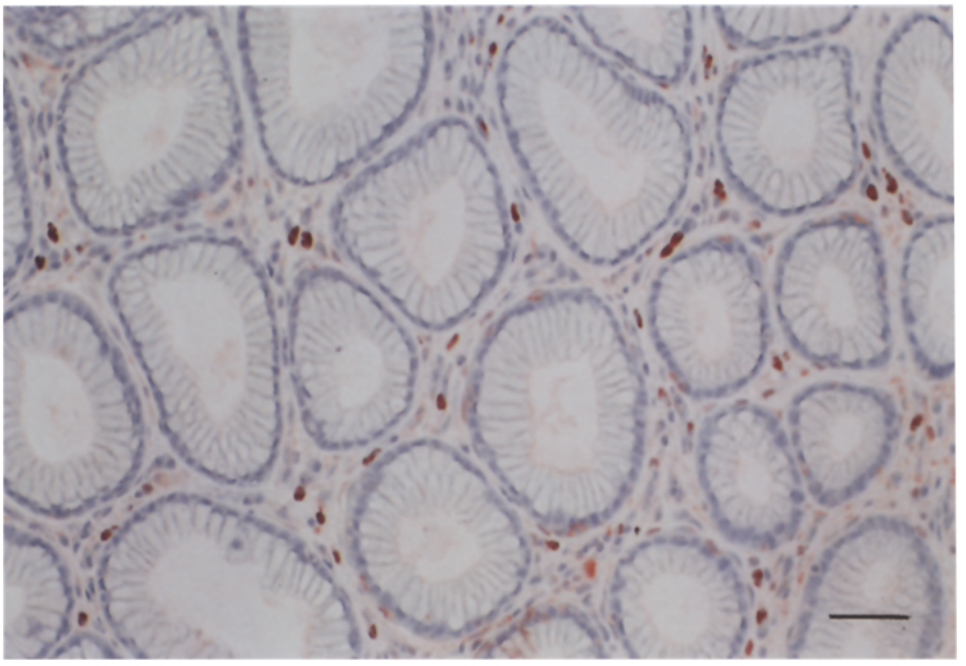


Fig. 1 Contrast to strong arginase immunoreactivity in infiltrated macrophage used as an internal positive control, no arginase immunoreactivity was found in normal gastric mucosal cells

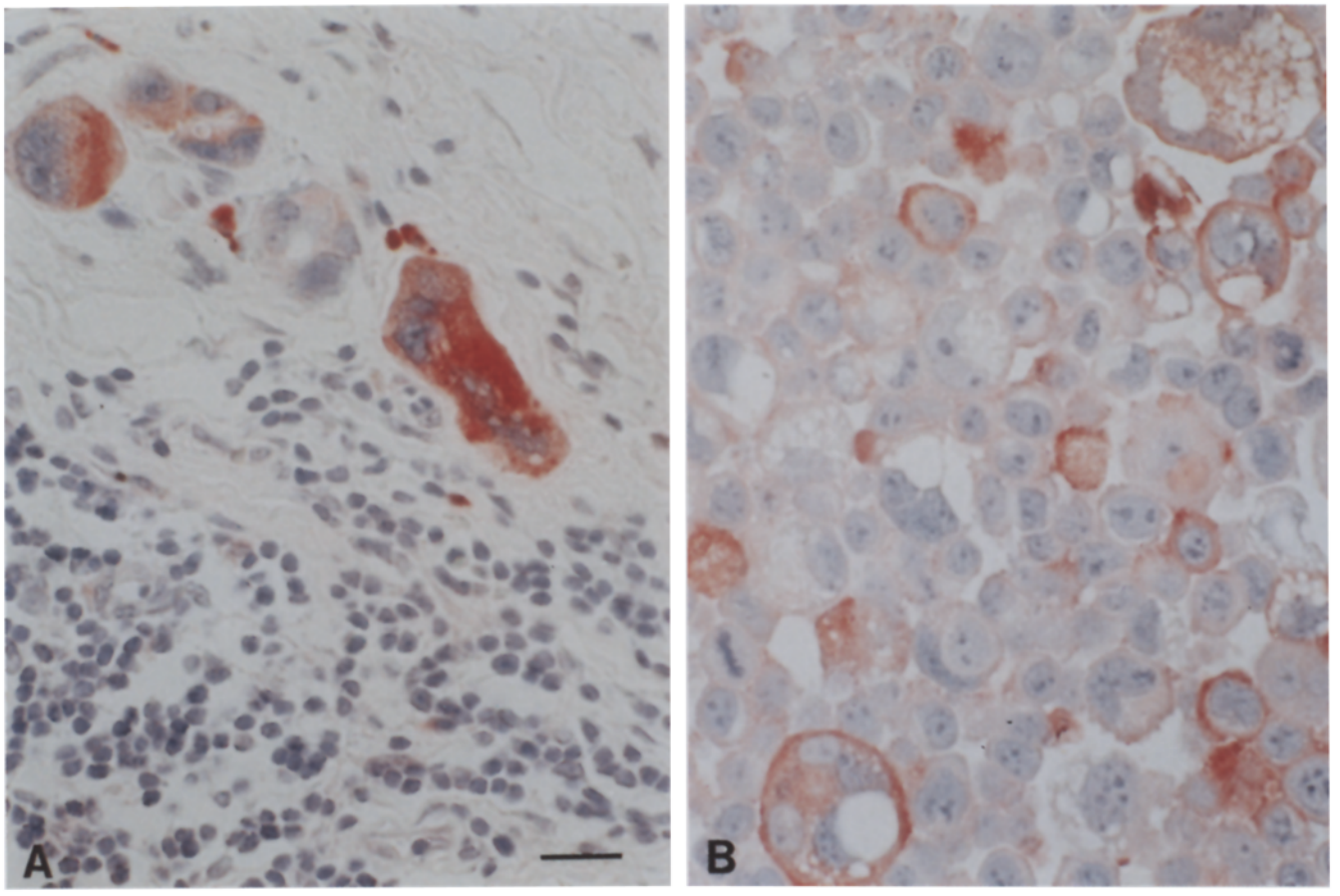
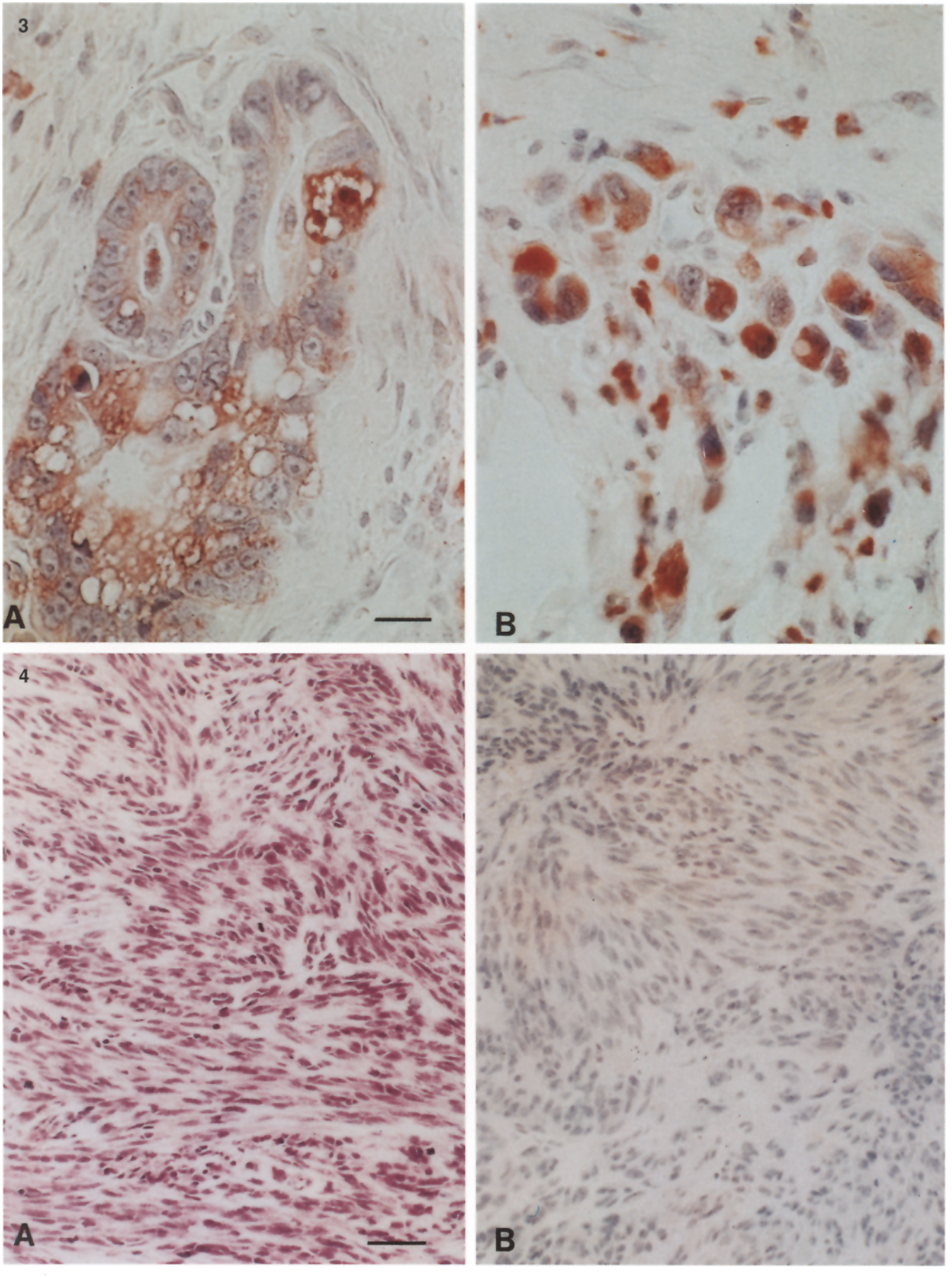


Fig. 2 **A** Arginase immunoreactivity with fine reddish-brown particles was present exclusively in the cytoplasm, and not in the nucleus (original magnification × 400) in a cancer cell. **B** Positive arginase immunoreactivity noted in a gastric cancer cell line KATO-III (original magnification × 400). *Scale bar* 20 μm

Source of antibody

The highly purified human arginase used as an antigen in antibody production was prepared in a similar way to that reported for murine arginase [6]. Arginase 500 μg in 1 ml complete Freund adjuvant (Difico Laboratories, Detroit, Mich.) was intradermally in-


3
A
B
4
A
B

jected to rabbits at multiple loci. Two weeks later, arginase 250 μg in 1 ml incomplete Freund adjuvant was similarly injected on two occasions 1 week apart. Serum was collected 1 week after the last booster.

The anti-human arginase polyclonal antibody was purified from rabbit antiserum by passing through an affinity column packed with human arginase-conjugated Sepharose-4B [18].

Immunohistochemical staining

Arginase in gastric tissues was localized with the avidin–biotin–peroxidase complex (ABC) technique of Hsu et al. [5]. All reagents were obtained from Vector Laboratories (Calif.). Briefly, the rehydrated tissue sections were first treated with 3% hydrogen peroxide and normal horse serum to remove endogenous peroxidase activity and to reduce nonspecific background staining. The tissue sections were incubated with rabbit anti-human arginase antiserum at 1:32 dilution at 25°C for 1 h in a moist chamber. The tissue sections were then treated with biotin-labelled goat anti-rabbit IgG antibodies, 50 μl in 10 ml phosphate-buffered saline (PBS), followed by avidin–biotin peroxidase complex (ABC). Fresh ABC was made by incubating 10 μl avidin and 10 μl biotin peroxidase in PBS for 30 min before use.

ABC staining for negative controls was carried out by omission of the primary antiserum or replacement of the primary antiserum by nonimmune normal rabbit serum. Normal liver tissue sections with known arginase content were also run concurrently for positive controls. The macrophages were identified by a lysozyme stain, and the adjacent section was processed for arginase immunocytochemical staining. The sections adjacent to those selected for ABC staining were stained with haematoxylin-eosin for comparison.

The distribution of positive immunoreactivity for arginase in tissue specimens was evaluated by a semi-quantitative system to calculate the percentage of positive cells in nonoverlapping microscopic fields and estimated within the following arbitrary ranges: –, no positive cells; +, 1–25%; ++, 26–75%; +++, more than 75%.

Statistical analysis

The data were expressed as mean ± SD, and the statistical significance was calculated according to the *t*-test and the chi-square test. $P<0.05$ was considered significant.

Results

Normal tissue from stomach

Immunoreactivity of arginase in normal stomach showed fine reddish-brown particles. In the paraffin sections of normal gastric tissue, no arginase immunoreactivity was observed in mucosal cells (Fig. 1) or in muscular and serosal tissues. As expected, arginase was found to be present in macrophages, erythrocytes, and leucocytes. Macrophages with strong arginase immunoreactivity infiltrating both normal and cancerous gastric tissues provided an internal positive control for arginase. A relatively weak immunoreactivity was noted in erythrocytes and leucocytes. However, there was no arginase immunoreactivity in plasma cells, lymphocytes or benign gastric ulcers.

Table 2 Summary and comparison of the clinicopathological features of gastric carcinomas regarding positivity or negativity for arginase expression

	Arginase expression	
	Positive	Negative
No. of cases	28	17
Age (years), mean	68.2±10.1	61.7±13.1
Sex		
Male	23	13
Female	5	4
Tumour size (cm)	6.6±2.5	6.9±3.3
Location		
Upper stomach	2	2
Middle stomach	10	4
Lower stomach	16	11
Gross type		
Borrmann 0, I, II	6	4
Borrmann III, IV	22	13
Grading		
Well differentiated	2	1
Moderately differentiated	7	3
Poorly differentiated	19	13
Lauren classification		
Intestinal type	27[a]	13
Diffuse type	22	12
Growth pattern		
α, β	9	6
γ	19	11
Clinical stage		
I, II	9	6
III, IV	19	11

[a] Both intestinal and diffuse types in the same cancer tissue had similar arginase immunoreactivity; the case numbers differ because some cancer tissues had intestinal (or diffuse) type alone

Cancer cell lines and cancerous tissue from stomach

At high power (× 400), the arginase immunoreactivity was observed exclusively in the cytoplasm (Fig. 2A). Arginase immunoreactivity was demonstrated in 62.2% (28/45) of stomach adenocarcinomas and was heterogeneous in type; the intensity varied from weak to strong in different cells in the same cancer cell nest. There was no difference in the positive rate and percentage of arginase immunoreactivity between diffuse and intestinal types (Fig. 3A, B). The intensity of immunoreactivity at the invading edge of tumours and in lymphatic or vascular tumour emboli was equivalent to the intensity seen in the remaining tumour tissue. The presence of arginase in the cells of the tumours was not related to the clinicopathological features ($P>0.05$; Table 2).

Dysplastic glands adjacent to cancer lesions were observed in 35 (78%) specimens. Scattered arginase immu-

Fig. 3 Heterogeneity of arginase immunoreactivity in **A** intestinal-type (original magnification × 400) and **B** diffuse-type adenocarcinomatous tissues (original magnification × 400). *Scale bar* 20 μm

Fig. 4 **A** Increased mitotic figures in leiomyosarcoma shown on HE staining. **B** No arginase immunoreactivity was shown in leiomyosarcoma (original magnification × 200). *Scale bar* 40 μm

noreactivity was noted in 42.8% (12/28) of these dysplastic glands, but there was no arginase immunoreactivity in any case of the intestinal metaplasia, regardless of its type. No arginase immunoreactivity was observed in the dysplastic glands of 7 gastric carcinomas without immunoreactivity in the neoplastic cells.

Positive arginase immunoreactivity was observed in all three gastric cancer cell lines; the immunoreactivity was heterogenous in the cells of the same tumour line (Fig. 2B).

No immunohistochemical arginase immunoreactivity was seen in leiomyoma or leiomyosarcoma (Fig. 4A, B) of the stomach.

Discussion

This study shows that arginase is present in the majority of human gastric adenocarcinoma cells. We found a heterogeneous expression of arginase in 62.2% of adenocarcinoma tissues and all three gastric cancer cell lines. Scattered arginase immunoreactivity was noted in 42.8% of dysplastic glands adjacent to carcinomas with positive arginase immunoreactivity. In contrast, arginase was not observed in normal gastric mucosal gland, intestinal metaplasia, muscles, serosas, leiomyoma and leiomyosarcomas.

We have reported that gastric carcinomas possess higher arginase levels than the corresponding normal mucosal tissues [20, 21]. Arginase clearly exists in 62.2% of gastric adenocarcinomas and in gastric cancer cell lines that constitutively release arginase [23]. On the basis of these and our earlier observations, it would be reasonable to infer that the high arginase levels found in gastric adenocarcinomas are largely produced by gastric cancer cells. In contrast to adenocarcinoma, gastric tumours of muscular origin, such as leiomyomas and leiomyosarcoma, contained no arginine.

Previous studies have shown that arginase facilitates cancer spread [24]. Our current results have further demonstrated differential expression of arginase in different tumours and within the same lesion (heterogeneity shown in Fig. 3).

Although strong arginase expression is noted in macrophages or chronic inflammatory cells, there is no arginase immunoreactivity in the remaining mucosal glands in gastric ulcer. Previous studies have also failed to detect increased arginase levels in patients with peptic ulcer [23]. Our findings are apparently different from those of Porembska and Kedra [13], who reported elevated serum arginase activity in myocardial infarction and speculated that the necrotic tissue released arginase. However, the release of arginase from macrophages in tissue necrosis may account for the elevated serum arginase in some inflammatory conditions.

It is generally recognized that arginase is abundant in the human liver and is present in traces in other organs, such as erythrocytes, kidney, brain, intestine [3], and colon [11]. Like Multhaupt et al. [12], we have found by immuno-histochemical staining that arginase is localized in the cytoplasm of hepatocytes (picture not shown). However, our current study did not show arginase staining in normal gastric mucosal glands. Previously we have found detectable arginase from cryostat sections of normal gastric mucosal glands [20], and this change may have resulted from the loss of some antigenicity of arginase during the wax-embedding process. Nevertheless, consistently elevated levels of arginase were found in carcinomas regardless of any variation in the sample preparation.

Acknowledgements This study was supported by a grant from National Science Council of the Republic of China (NSC-77-0412-B075-61).

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