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Existence of Lipoprotein Lipase in Human Sarcomas and Carcinomas

Kenshi Sakayama,[1] Hiroshi Masuno,[2,4] Tatsuhiko Miyazaki,[3] Hideo Okumura,[1] Taihoh Shibata[1] and Hiromichi Okuda[2]

Departments of [1]Orthopaedic Surgery and [2]Medical Biochemistry, and [3]Second Department of Pathology, School of Medicine, Ehime University, Shigenobu, Onsen-gun, Ehime 791-02

Aqueous extracts of acetone/ether powders of surgically obtained specimens of human tumors hydrolyzed ^{3}H-labeled triolein in a dose-dependent manner. The lipolytic activity in these extracts was inhibited by anti-lipoprotein lipase (LPL) IgG dose-dependently, 25 μg of anti-LPL IgG causing 95% inhibition of the activity. Thus, LPL accounts for most of the lipolytic activity in extracts of acetone/ether powders of the tumors. All sarcomas and carcinomas examined contained LPL activity. Western blotting showed that they gave a band corresponding to that of human adipose tissue LPL (M_r=57,000). Immunocytochemical studies showed that LPL was present in cultured human osteosarcoma cells and distributed throughout the cells. We determined the proliferating cell nuclear antigen (PCNA)-labeling index as an indicator of the proliferative activity of tumor cells and measured LPL activity in extracts of tumors in areas corresponding to those used for determining the PCNA-labeling index. In malignant fibrous histiocytomas, the PCNA-labeling index in area a, which corresponds to the subcapsular region, was higher than that in area b, which corresponds to the central region. The LPL activity in area a was 10 times that in area b. In rectal cancer, the index in area c, which corresponds to the subserosal region, was higher than that in area d, which corresponds to the submucosal region. The LPL activity in area c was 1.9 times that in area d. These findings indicate heterogeneity in the distributions of LPL activity within tumors and higher levels of LPL activity in tumors that are proliferating actively.

Key words: Lipoprotein lipase — Sarcoma — Carcinoma — Proliferating cell nuclear antigen — Human

Proliferating tumor cells need a large amount of energy for growth. They can utilize ATP produced during conversion of glucose into lactate for growth,[1] while *in vitro*[2-4] and *in vivo*[5] experiments have shown that fatty acids (FAs) are also utilized as energy sources. These FAs are supplied to cells in 3 forms: as FA synthesized *de novo* in the cells, as albumin-bound free fatty acid (FFA) in the circulation, and as esterified FA in chylomicrons and very low density lipoprotein (VLDL) in the circulation. Albumin-bound FFA is taken up directly by the cells, whereas esterified FAs are hydrolyzed to FFA and monoacylglycerol by lipase, and the FFA formed is taken up by the cells.

Lipoprotein lipase (LPL) plays a major role in the hydrolysis of triacylglycerol (TG) in chylomicrons and VLDL in the circulation. This enzyme is synthesized in parenchymal cells of extrahepatic tissues, mainly the adipose tissue, heart and mammary glands, and is attached to heparan sulfate proteoglycan on the luminal surface of capillaries in tissues where it acts.[6-8] LPL is involved in not only clearance of TG from the circulation but also uptake by the tissues of FFAs derived from TG. Thus LPL provides FFAs for energy metabolism or storage in the cells. However, there has been no report on the existence of LPL in surgically obtained specimens of human tumors.

Recently, proliferating cell nuclear antigen (PCNA), which is a nonhistone nuclear protein of M_r=36,000 present in the nuclei of proliferating cells,[9-12] has been reported to be useful as a marker of cell proliferation. Robbins *et al.*[13] reported that PCNA positivity in human solid tumors correlated with mitotic activity and the grade of tumors.

The aim of this study was to demonstrate the existence of LPL in surgically obtained specimens of human sarcomas and carcinomas. We also showed immunocytochemically that cultured human osteosarcoma cells synthesized active LPL. In addition, to assess the role of LPL in tumor growth, we determined the PCNA-labeling index, as an indicator of the proliferative activity of cells, of tumors in the area corresponding to that in which LPL activity was measured.

MATERIALS AND METHODS

Materials Chicken antiserum to bovine LPL and chicken anti-LPL IgG were gifts from Drs. Thomas Olivecrona and Gunilla Bengtsson-Olivecrona, Department of Physiological Chemistry, University of Umeå, Umeå, Sweden.

[4] To whom all correspondence should be addressed.

Solution A, used for aqueous extraction of acetone/ether powders, consisted of 50 m*M* NH_3/NH_4Cl and 20 μg/ml heparin, pH 8.2. Solution B, used when the nitrocellulose was incubated with antibodies, consisted of 20 m*M* Tris and 137 m*M* NaCl. Solution C, used when the nitrocellulose was washed, consisted of 0.1% Tween 20 in solution B.

Preparation of acetone/ether powders of tumors Specimens of tumors were obtained at surgery, promptly immersed in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5 μg/ml insulin and brought to the laboratory within 10 min. Samples of 500 mg of tumors were homogenized with a Teflon-glass homogenizer in 1.5 ml of solution A supplemented with 2% bovine albumin at 10°C. The homogenates were sonicated briefly at 0°C and then used to prepare acetone/ether powders as described previously.[14, 15] Acetone/ether powders were prepared within 20 min after excision of tumors.

Assay of LPL activity LPL activity was measured in aqueous extracts of acetone/ether powders of tumors with tri[9,10(n)-^{3}H]oleoylglycerol as the substrate in the presence of heat-inactivated (56°C, 10 min) fasted rat serum.[14, 15] One milliunit of lipolytic activity was defined as that releasing 1 nmol of fatty acid/min at 37°C.

Immunoinhibition study Aliquots of extracts of acetone/ether powders of tumors were mixed with diluted chicken anti-LPL IgG solution or chicken IgG solution, and the mixtures were allowed to stand for 2 h at 0°C. Then, the lipolytic activity in the mixture was measured.

Immunocytochemical study A human osteosarcoma cell line, osteosarcoma Takase (OST),[16] was kindly given by Dr. Katsuro Tomita, Department of Orthopaedic Surgery, School of Medicine, Kanazawa University, Kanazawa. OST cells were cultured in RPMI-1640 medium in chamber slides (Nunc, Inc.). A fluorescent double antibody technique was used for immunolocalization of LPL in cultured OST cells. The primary reactant was mouse monoclonal antibody to LPL (Oncogene Science, Inc.) and the secondary reactant was rhodamine-labeled anti-mouse immunoglobulins (Dako Co.). The cells were fixed with absolute methanol and then made permeable with 0.1% saponin and 0.75 m*M* bovine albumin in Dulbecco's phosphate-buffered saline before being incubated with the antisera.

Western blotting Aliquots of extracts from acetone/ether powders of tumors were mixed with an equal volume of 0.125 *M* Tris-HCl buffer (pH 6.8) containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue and heated for 5 min at 95°C. Proteins in the extracts were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Laemmli type system[17] with 10% acrylamide resolving gel and 3% acrylamide stacking gel. The proteins separated were then transferred electrophoretically to nitrocellulose and blotted. Nonspecific binding was blocked by incubating the nitrocellulose in solution C containing 5% dried milk for 1 h. The blots were washed with solution C, and then incubated for 1 h with chicken antiserum to bovine LPL at a dilution of 1:6,000 in solution B. The blots were washed with solution C and exposed for 1 h to rabbit anti-chicken IgG (Pel-Freez) at a dilution of 1:20,000 in solution B. They were then washed with solution C, incubated for 1 h with ^{125}I-protein A (200,000 cpm/ml), washed with solution C, and exposed to Kodak X-Omat film.

PCNA-labeling index and number of macrophages Tumors were fixed for 24–48 h in 10% formalin and embedded in paraffin by standard procedures. After deparaffinization, the sections were incubated for 10 min with nonimmune goat serum to block nonspecific binding and then incubated for 1 h at room temperature with the primary antibody to PCNA (Dako Co.). PCNA-positive nuclei were detected with an SAB kit (Dako Co.). Cells were considered to contain PCNA when their nucleus stained red. The PCNA-labeling index was evaluated by determining the percentage of positive nuclei present in approximately 200 cells. Values are shown as means ± SD for 5–6 determinations.

Macrophages were counted in the formalin-fixed, paraffin-embedded tumor tissue sections described above.

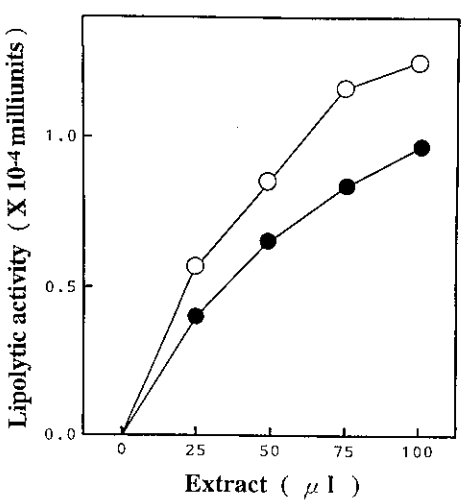

Fig. 1. Lipolytic activities in a malignant fibrous histiocytoma and rectal cancer. Samples of 500 mg of tumors were homogenized with a Teflon-glass homogenizer in 1.5 ml of solution A containing 2% bovine serum albumin at 0°C. The homogenates were sonicated briefly at 0°C and aliquots of the homogenates were used to make acetone/ether powders. Lipolytic activities were measured with the indicated volumes of aqueous extracts of the powders. (○), rectal cancer; (●), malignant fibrous histiocytoma.

After deparaffinization, the sections were incubated for 10 min with nonimmune goat serum to block nonspecific binding and then incubated for 16 h at 4°C with monoclonal mouse anti-human macrophages (Dako Co.). Cells that reacted with the primary antibody were detected with an SAB kit. Numbers of macrophages present in 10 mm^2 areas of sections were counted.

DNA measurement DNA was measured fluorometrically by the method of Hinegardner[18] with calf thymus DNA as a standard.

RESULTS

Existence of LPL in human tumors The lipolytic activity in extracts of acetone/ether powders of surgically obtained specimens of tumors was measured. Fig. 1 shows the dose-dependency of lipolytic activities of extracts of a malignant fibrous histiocytoma and rectal cancer. Both lipolytic activities increased with increases in the volume of the extract.

Next, the effect of anti-LPL IgG on the lipolytic activity was examined. Fig. 2 shows that anti-LPL IgG inhibited the activity dose-dependently, 25 μg of anti-LPL IgG causing over 95% inhibition. Chicken IgG had no effect on the activity. These results indicate that LPL accounted for most of the lipolytic activity in extracts of acetone/ether powders of surgically obtained tumor specimens.

The LPL activities in extracts of acetone/ether powders of surgically obtained specimens of sarcomas and carcinomas were measured (Table I). Osteosarcomas contained very high LPL activity. Malignant fibrous histiocytomas also contained LPL activity, but their activities were very variable. A bladder carcinoma had the lowest activity. These results indicate that human sarcomas and carcinomas contained active LPL.

To determine whether LPL protein was present in the tumors, we subjected the extracts of acetone/ether

Table I. LPL Activity in Various Human Tumors

Tumors	LPL activity (mU/mg DNA)	
Osteosarcomas	26.1±5.3[a]	(3)[b]
Malignant fibrous histiocytomas	3.1±4.6	(3)
Malignant melanomas	1.9±0.3	(3)
Chondrosarcoma	2.4	(1)
Rectal cancers	3.2±1.4	(2)
Gastric cancers	5.0±0.3	(2)
Bladder carcinoma	0.4	(1)
Mandibular carcinoma	3.0	(1)

Samples of 500 mg of tumors were homogenized with a Teflon-glass homogenizer in 1.5 ml of solution A containing 2% bovine albumin at 10°C. The homogenates were sonicated briefly at 0°C and then used to prepare acetone/ether powders. LPL was extracted from the acetone/ether powders with solution A as described previously.[15] LPL activity in aliquots of extracts was assayed in duplicate.

a) Mean±SD.

b) Numbers in parentheses, numbers of tissue samples.

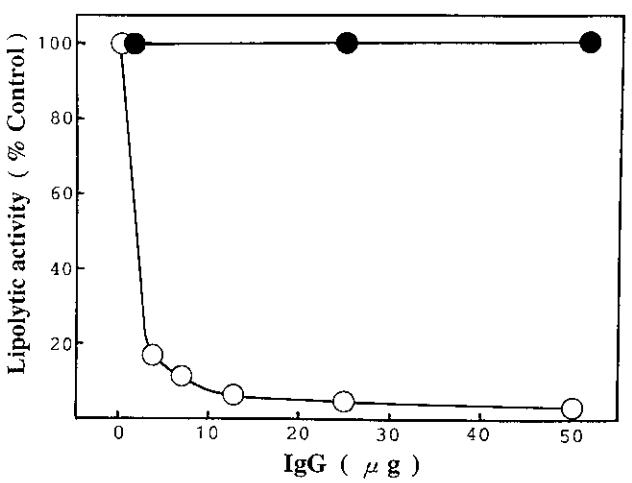

Fig. 2. Immunoinhibition of LPL activity of an osteosarcoma. A mixture of 25 μl of the extract of an acetone/ether powder of osteosarcoma was incubated for 2 h with either anti-LPL IgG (○) or nonimmune chicken IgG (●) at the indicated concentration at 0°C, and lipase activity was assayed as described under "Materials and Methods." The initial lipolytic activity in the extract was 24.1 mU/ml.

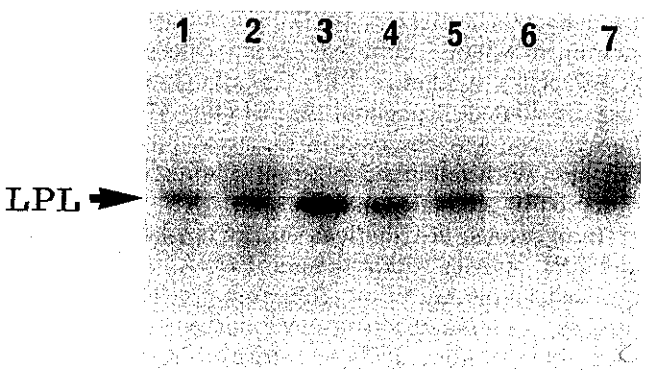

Fig. 3. Western blot of LPL in tumors. The extracts of acetone/ether powders of tumors and human adipose tissue were prepared as described under "Materials and Methods" and applied to a gel. After transfer to a nitrocellulose membrane, the separated proteins were blotted with chicken antiserum to bovine LPL. All blots were then exposed to rabbit anti-chicken IgG and ^{125}I-protein A as described under "Materials and Methods." Lanes 1 and 7, human adipose tissue; lane 2, malignant fibrous histiocytoma; lane 3, osteosarcoma; lane 4, rectal cancer; lane 5, mandibular carcinoma; lane 6, bladder carcinoma.

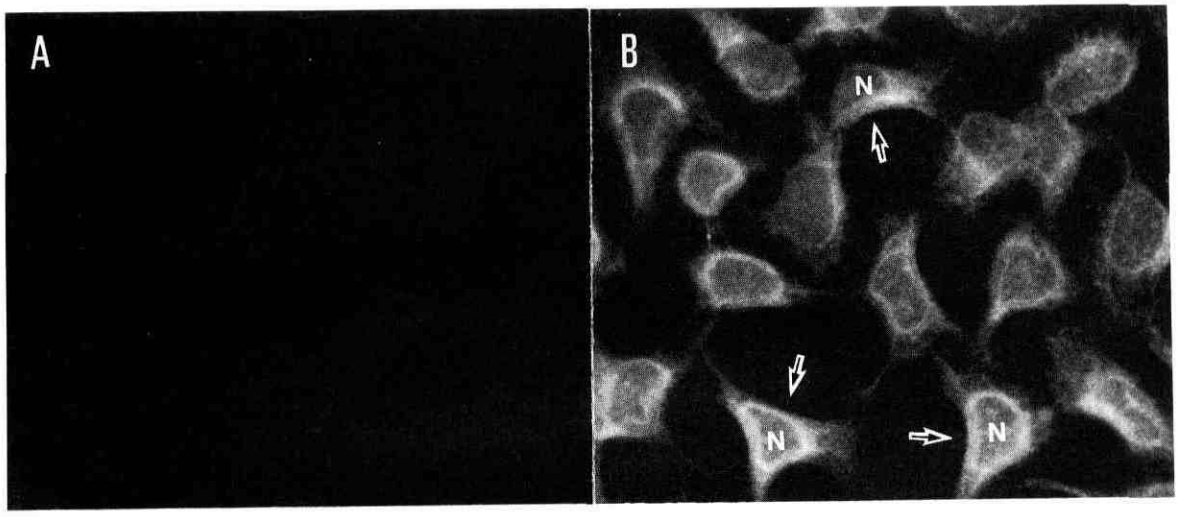

Fig. 4. Light micrographs showing immunofluorescent localization of LPL in cultured OST cells. (A) As a control, mouse nonimmune serum was used as the primary antibody. (B) LPL was localized in cells using mouse monoclonal antibody to LPL as the primary antibody. Arrow, LPL; N, nucleus; ×400.

powders of surgically obtained tumor specimens to SDS-PAGE, transferred them to nitrocellulose, and examined them by blotting with antiserum to bovine LPL. As shown in Fig. 3, in the extracts of the tumors, the antiserum identified a band that co-migrated with human adipose tissue LPL. Our previous findings showed that the molecular weight (M_r) of LPL in human adipose tissues was 57,000.[19] These results indicate that the tumors contained LPL protein of $M_r = 57,000$.

Immunocytochemical study Immunocytochemically, we examined whether cultured human osteosarcoma cells (OST cells) synthesize LPL. Immunofluorescent LPL was distributed throughout the cell (Fig. 4B). Very light fluorescence was observed in the areas surrounding the nucleus. With nonimmune serum, fluorescence was not observed in cells (Fig. 4A).

Heterogeneous distributions of PCNA-labeling index and LPL activity within tumors PCNA, which is accumulated in the nuclei of proliferating cells,[9-12] is often used as an immunohistochemical marker of proliferating cells. Fig. 5 shows PCNA-positive cells in sections of tumors embedded in paraffin. We determined the PCNA-labeling index by counting PCNA-positive cells in the tumor (Table II). In the malignant fibrous histiocytoma shown in Fig. 5A, areas a and b correspond to the subcapsular and central regions, respectively. Area b contained much necrotic tissue (Fig. 5b, arrowhead). The PCNA-labeling index in area a was higher than that in area b, indicating that tumor cells proliferated more actively in area a. In the rectal cancer shown in Fig. 5B, areas c and d correspond to the subserosal and submucosal regions, respectively. The index in area c was higher than that in area d, indicating that tumor cells proliferated more actively in area c.

Table II. PCNA-labeling Indices and LPL Activities in Different Regions of Tumors

Tumors	Area[a]	PCNA-labeling index (%)	LPL activity (mU/mg DNA)
Malignant fibrous histiocytoma	a	48.1±1.9[b]	8.4
	b	40.6±3.1	0.8
Rectal cancer	c	57.5±5.0	8.5
	d	36.9±3.5	4.4

The PCNA-labeling index was evaluated by determining the percentage of PCNA-positive cells among the cells present in the area described in Fig. 5. Tumor tissue in the region corresponding to the area used for determining the PCNA-labeling index was used to prepare an acetone/ether powder for measuring LPL activity.

a) These areas correspond to those described in Fig. 5.

b) Mean±SD for 5–6 determinations.

The LPL activities were measured in extracts of the tumors in the areas corresponding to those in which the PCNA-labeling indices were determined (Table II). The LPL activity in the extract of area a of malignant fibrous histiocytoma was 10 times that in area b. The activity in the extract of area c of rectal cancer was 1.9 times that of area d.

Finally, we determined immunohistochemically the numbers of macrophages present in the areas described above. Areas a and b of malignant fibrous histiocytoma

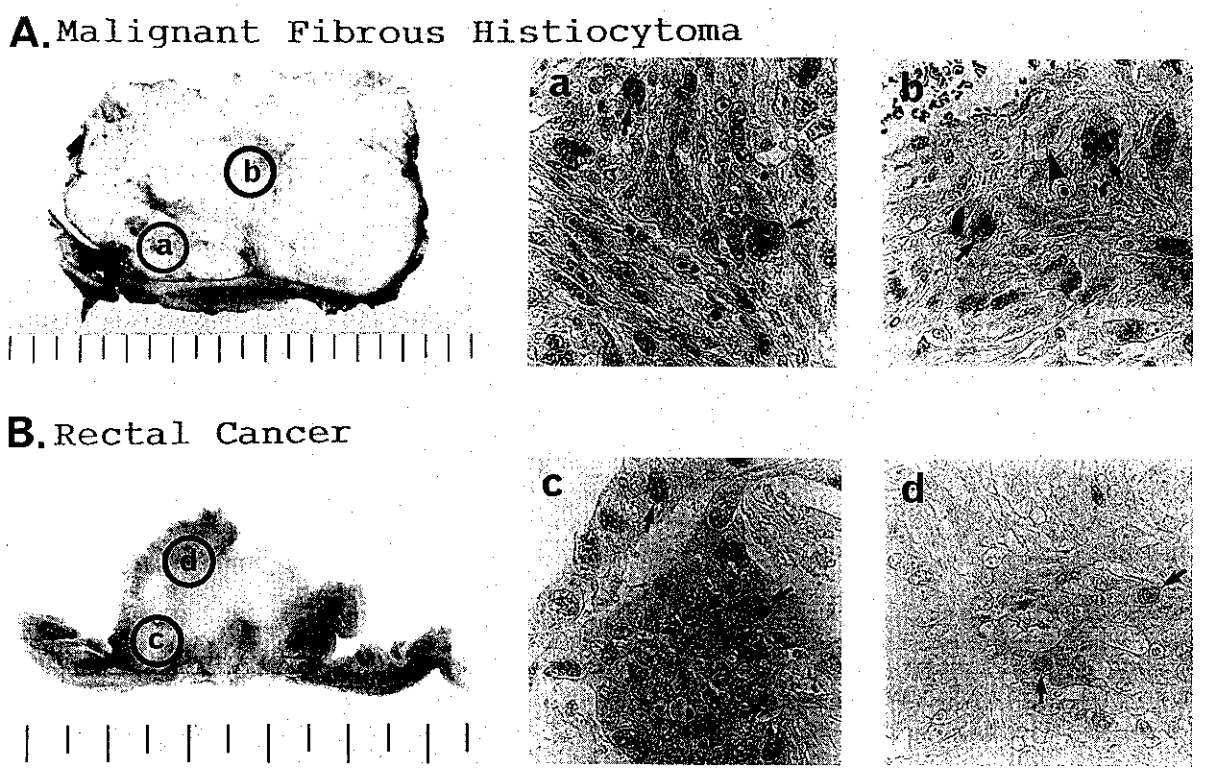

Fig. 5. Macroscopic appearances of malignant fibrous histiocytoma (A) and rectal cancer (B). Photographs (a–d) show the immunolabeling of PCNA; a, immunolabeling of area a in the malignant fibrous histiocytoma (A); b, immunolabeling of area b in the malignant fibrous histiocytoma (A); c, immunolabeling of area c in the rectal cancer (B); d, immunolabeling of area d in the rectal cancer (B). Arrows indicate PCNA-positive cells. The arrowhead indicates a necrotic region.

contained 241 and 240 macrophages/10 mm^2, respectively, and areas c and d of rectal cancer contained 329 and 261 macrophages/10 mm^2, respectively.

DISCUSSION

TG in chylomicrons and VLDL in the circulation is hydrolyzed to FFA and monoacylglycerol by LPL. This FFA is transferred across the capillary wall to cells in extrahepatic tissues where it is used for synthesis and storage of phospholipids and TG.[6, 20] Part of the FFA is oxidized in mitochondria to supply energy for cells of the body.

Ascites fluid formed in animals with ascites tumors contains large amounts of VLDL.[21–23] Brenneman and Spector[21] showed, using ^{14}C-labeled triolein, that FAs derived from VLDL-TG were incorporated into TG and phospholipids and oxidized to CO_2 in mouse Ehrlich ascites tumor cells. But, they did not observe release of lipase activity from Ehrlich cells. By contrast, Balint and Holczinger[24] found that ascites fluid of mice with Ehrlich cells contained LPL activity.

In the present studies, we found that human sarcomas and carcinomas contained active LPL of $M_r = 57{,}000$ (Table I and Fig. 3). The LPL activity was distributed heterogeneously within tumors and it was high in the area containing actively proliferating cells (Table II). The LPL activity in the area containing much necrotic tumor tissue was very low (Fig. 5b). Thus, the level of LPL activity correlated with tumor growth. These results suggest that FFA produced from circulating TG by LPL could be utilized as energy for proliferation of tumor cells.

At least three possibilities for the origin of LPL in surgically obtained specimens of human tumors may be considered. One is that the tumor cells themselves synthesize LPL. Our immunofluorescence findings showed that LPL was present in cultured OST cells (Fig. 4). In addition, the extract of acetone/ether powder of OST cells hydrolyzed ^3H-labeled triolein in a dose-dependent manner (data not shown). These results indicate that cultured human osteosarcoma cells synthesize active LPL. Another possibility is that macrophages present in tumors synthesize and secrete LPL. Human monocyte-

derived macrophages and rabbit alveolar macrophages synthesize and secrete LPL.[25-27] Tajima *et al.*[28] reported that the human monocytic leukemia cell line THP-1, which is induced to differentiate into macrophages by the addition of 4β-phorbol 12β-myristate 13α-acetate (TPA), synthesizes LPL in the presence of TPA. Our immunohistochemical study showed that area a in malignant fibrous histiocytomas contained the same number of macrophages as area b, whereas its LPL activity was 10 times that of area b (Table I). Moreover, the LPL activity in osteosarcomas was very high, but the number of macrophages in osteosarcomas (242/10 mm^2) was almost the same as that in malignant fibrous histiocytomas and rectal cancers. These findings suggest that LPL in surgically obtained specimens of human tumors does not result from secretion by macrophages. A third possibility is that LPL synthesized by parenchymal cells in extrahepatic tissues is transferred to, and becomes located in capillaries in tumors. To our knowledge, however, there has been no report of any such phenomenon. Thus, the origin of LPL in surgically obtained specimens of human tumors is probably the tumor cells themselves.

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