in nasopharyngeal carcinoma, other head and neck neoplasms and control groups. *J Natl Cancer Inst* 1970, **44**, 225–231.
18. Young LS, Dawson CD, Clark D, *et al.* Epstein–Barr virus gene expression in nasopharyngeal carcinoma. *J Gen Virol* 1988, **69**, 1051–1065.
19. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. Expression of Epstein–Barr virus latent gene products in tumour cells of Hodkgin's disease. *Lancet* 1991, **337**, 320–322.
20. Herbst H, Steinbrecher E, Niedobitek G, *et al.* Distribution and phenotype of Epstein–Barr virus harboring cells in Hodgkin's disease. *Blood* 1992, **80**, 484–491.
21. Schulley TB, Cross SM, Borrow P, Cooper DA. Prevalence of antibodies to Epstein–Barr virus nuclear antigen 2B in persons infected with the human immunodeficiency virus. *J Inf Dis* 1988, **158**, 186–192.

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A Cross-linking Assay Allows the Detection of Receptors for the Somatostatin Analogue, Lanreotide in Human Breast Tumours

Grégoire Prévost, Philippe Provost, Valérie Sallé, Monique Lanson and François Thomas

Hypothalamic somatostatin and its synthetic analogues inhibit the cell growth of several tumour models. The somatostatin analogue lanreotide (somatuline or BIM23014C) inhibits both the *in vivo* and *in vitro* cell growth of various mammary tumours. In order to evaluate the presence of receptors for lanreotide in breast tissue, samples from 41 female and 2 male patients were analysed by a cross-linking assay. All the samples examined possessed at least one subtype of lanreotide binding polypeptide, however, different polypeptide patterns were observed. The two major complexes had molecular weights of 57 kD and 42 kD. The previously demonstrated antiproliferative activity of lanreotide and the high percentage of positive tumours supports the use of lanreotide in clinical trials. However, the role of each receptor subtype in the control of breast cell proliferation requires further characterisation.
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INTRODUCTION

HYPOTHALAMIC SOMATOSTATIN [1] and its analogues [2] inhibit both the *in vivo* and *in vitro* cell growth of various human cell lines including small cell lung cancer, pancreatic carcinoma, prostatic carcinoma and breast carcinoma [3–7]. The antiproliferative effect of these molecules is the result of their direct activity on tumour growth and their inhibition of hormone secretion (growth hormone, prolactin, and insulin-like growth factor) [8]. Multiple subtypes of the somatostatin receptor have been observed by chemical cross-linking in various cells such as pituitary adenomas [9] or breast cancer cell lines [3]. The molecular heterogeneity of the somatostatin binding polypeptides has been elucidated by a quantitative autoradiographic study of the rat brain and the adenohypophysis demonstrating the presence of different subtypes of receptors in these tissues [10]. Recently, more than two different genes coding for somatostatin binding polypeptides have been cloned demonstrating the existence of various somatostatin receptors [11]. The somatostatin analogue, Lanreotide (somatuline or BIM23014C) [12] is an efficient *in vivo* and *in vitro* antiproliferative agent in several mammary models. Between 15 and 90% of breast tumours express somatostatin receptors [3, 8, 13, 14]. This variation may be due to differences in labelled ligands and in the methodology used for their detection (cryostat-section autoradiography, chemical cross-linking, *in vivo* scintigraphy, filtration-retention). The heterogeneity of somatostatin receptor distribution in breast biopsy specimens may also account for this difference [15]. In order to evaluate the potential direct activity of the somatostatin analogue lanreotide in breast tumours, lanreotide receptors have been analysed by the cross-linking assay in 43 surgical samples (patients: 41 females and 2 males).

Correspondence to G. Prévost.
G. Prévost, P. Provost and V. Sallé are at the Institut d'Oncologie Cellulaire et Moléculaire Humaine, Laboratoire de Biophysique et Pharmacologie des Biosignaux, Université Paris-Nord, 129 route de Stalingrad, Bobigny, 93000, France; M. Lanson is at the Laboratoire de Biologie des tumeurs, 2, bis Boulevard Tonnelle, 37032, Tours, France; and F. Thomas is at the Ipsen-Biotech, 15, rue Cambronne, 75737, Paris, France.
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MATERIALS AND METHODS

Materials

The somatostatin analogue lanreotide (BIM-23014C or somatuline) was provided by Ipsen-Biotech (Paris, France). The labelled peptide [^{125}I] lanreotide (592–740 GBq/mmol) was obtained from F. Dray and C. Tiberghien (Hospital Cochin, Paris, France). Ethylene glycol-bis (succinimidyl succinate) (EGS) was obtained from the Sigma Chemical Co. (France).

Tumour tissue samples

Specimens were obtained from 43 patients who had surgery for breast cancer in the Department of Gynecology (Hospital

Bretonneau, Tours, France). 2 patients (cases 44 and 72) received prior treatment. Frozen tissues were pulverised and homogenised in 10 mmol/l Tris–HCl, pH 7.8, 1.5 mmol/l EDTA, 10 mmol/l ammonium molybdate, 1.2 mmol/l monothioglycerol and 10% glycerol. Cytosolic fraction was separated from membrane enriched pellet by centrifugation (60000 *g* for 1 h at 4°C). Oestrogen and progesterone receptor assays were performed on the cytosolic fraction and lanreotide receptors on the pellet.

Cross-linking assays of the receptors for the somatostatin analogue: lanreotide

The membrane-enriched pellet was resuspended at 1 g per ml of cold buffer: 20% glycerol, 20 mmol/l Tris–HCl pH 7.4, 1 mmol/l phenylmethanesulphonyl fluoride (PMSF) and stored at −20°C. The protein content was determined by a colorimetric method (Sigma kit) and then diluted at 1 mg per ml in binding buffer 20 mmol/l Tris–HCl pH 7.4, 5 mmol/l $MgCl_2$, 1 mmol/l PMSF. 100 µl of the protein solution with 50 µl of [^{125}I]

Table 1. ^{125}[*I*]*Lanreotide binding polypeptides and receptors for oestrogen and progesterone in* 43 *breast tumours*

Tumour type	ER (fmol/mg)	PR (fmol/mg)	Age (years)	Lanreotide receptors (kD) 100	57	42	27
IDC	12	0	32	0	+	0	0
IDC	64	60	39	0	++	+	0
IDC	161	163	40	0	+	+	0
IDC	0	0	41	0	++	+	0
IDC	215	633	43	0	+	0	0
IDC	453	665	45	0	+	0	0
IDC	155	48	45	+	++	+	+
IDC	402	244	45	0	++	0	0
IDC	6	35	46	0	+	+	+
IDC	12	336	46	0	+	+	0
IDC	313	33	51	0	+++	+++	0
IDC	24	27	51	0	++	0	0
IDC	179	179	56	0	+++	++	+
IDC	966	3	60	0	+	0	0
IDC	2500<	409	61	0	++	+	0
IDC	390	7	68	0	++	+	0
IDC	14	0	69	0	++	+	0
IDC	60	168	74	0	++	+	0
IDC	12	0	75	0	+	0	0
IDC	2000<	203	82	0	+	0	0
IDC	373	3	85	0	+	0	0
IDC	116	25	nd	0	+	0	0
ILC	13	66	26	0	+	++	0
ILC	247	220	37	0	++	++	0
ILC	11	4	44	0	++	+	+
ILC	47	16	50	0	++	++	0
ILC	345	12	53	0	+++	+	+
ILC	1477	23	70	+	+++	++	+
ILC	79	13	85	0	+	0	0
IC	89	2	53	0	+	0	0
Comedocarcinoma	2	5	46	0	+	0	0
Adenofibroma	69	172	nd	0	++	+	0
nd	86	118	31	0	++	+	0
nd	7	6	40	0	+	+	+
nd	96	374	60	0	++	0	0
nd	114	32	66	0	+	+	0
nd	85	34	76	0	+	+	0
nd	331	62	79	0	+	+	0
nd	53	122	83	0	++	+	+
nd	1493	747	85	0	++	+	0
nd	1044	126	86	0	+	0	0
nd	222	44	nd	0	++	+	0
nd	446	73	nd	0	+	+	+

For each sample, the detection of each band is estimated as following: 0: no band, +: weak band, ++: medium band, +++ large band. IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; IC = intraductal carcinoma; nd: not determined.

lanreotide (10^5 dpm) solubilised in 20 mmol/l Tris–HCl pH 7.4, 5 mmol/l $MgCl_2$, 1 mmol/l PMSF, 0.1% bovine serum albumin (BSA) were incubated at 22°C for 90 min. The cross-linking agent ethylene glycol bis(succinimidyl succinate) (10^{-4} mol/l) was added at 4°C for 15 min. The cross-linking step was stopped by addition of 50 µl of loading buffer: 125 mmol/l Tris–HCl pH 7.4, 10% sodium dodecyl sulphate (SDS), 50% glycerol, 20% β-mercaptoethanol, 0.025% bromophenol blue. Samples were heated at 95°C for 2 min and proteins were separated on 12.5% SDS polyacrylamide gel. Gels were dried and autoradiographed at −80°C.

Oestrogen and progesterone receptor assays

Oestrogen receptors (ER) and progesterone receptors (PR) were measured on the cytosolic fractions using enzyme immunoassay kits (Abbott Laboratories, Abbott Park, Illinois, U.S.A.). Values are expressed in fmol/mg of cytosolic proteins with a cut-off value corresponding to 20 fmol/mg.

RESULTS

Receptors for lanreotide were determined in the membrane-enriched pellets by a chemical cross-linking assay with the linkage agent ethylene glycol-bis (succinimidyl succinate)(EGS). Both ER and PR were measured by immunoenzyme assays in the cytosolic fractions. The results obtained from 43 surgical biopsy specimens (41 females and 2 males) are presented in Table 1.

All the analysed samples from female and male patients possessed at least one subtype of lanreotide binding polypeptide, however, different patterns were observed. The two major complexes observed were 57 and 42 kD. Small amounts of the 27 kD and 100 kD polypeptides were also detected in some cases. The partial reaction of cross-linking is brought about to preserve the specificity of the binding process and to prevent the formation of protein aggregates. As a result, the spot detected on the autoradiogram represents just part of the receptors linked to the ligand and not all the receptors present in the tissue. This limits the overall quantification of the total number of each subtype.

One autoradiogram obtained with nine samples is shown in Fig. 1. The BSA used as protein competitor was non-specifically bound to the labelled peptide and was partially detectable (65–75 kD) in this assay. The ER and PR status of each tumour and patient age are presented in Table 1. The receptor distribution observed was: 60.5% of ER+ PR+, 16.2% of ER+ PR−, 7% of ER− PR+ and 16.3% of ER− PR−. The mean age of the patients was 58 years (26–85 years).

DISCUSSION

The results obtained from this study demonstrate the existence of binding sites for the somatostatin analogue, lanreotide in human breast tumours. These receptors were detected by a cross linking assay in samples from 43 patients, both male and female.

The two major complexes (42 and 57 kD) correspond with those previously described in breast epithelial cell lines [3] and in breast fibroblastic cells. The 42 kD polypeptide may correspond with the molecular weight of the somatostatin receptors cloned by Yamada *et al.* [11]. The second polypeptide (57 kD) has the same molecular weight as that of the somatostatin receptor characterised in the normal rat pituitary [16].

In this study, lanreotide receptors were detected in all the breast tumours examined. The detection procedure was slightly modified in comparison with that used in the previous study, which revealed a lower percentage of lanreotide receptor-positive biopsy specimens studied in comparison with the present study [3]. In the present study, the separation of the free- and the covalently bound-ligand was carried out solely by electrophoresis without centrifugation. This prevents the loss of soluble proteins and reduces the time of manipulation. These modifications may explain the high percentage of positive samples identified and the presence of a new minor polypeptide: 100 kD. The percentage of positive biopsy samples observed has been compared with the

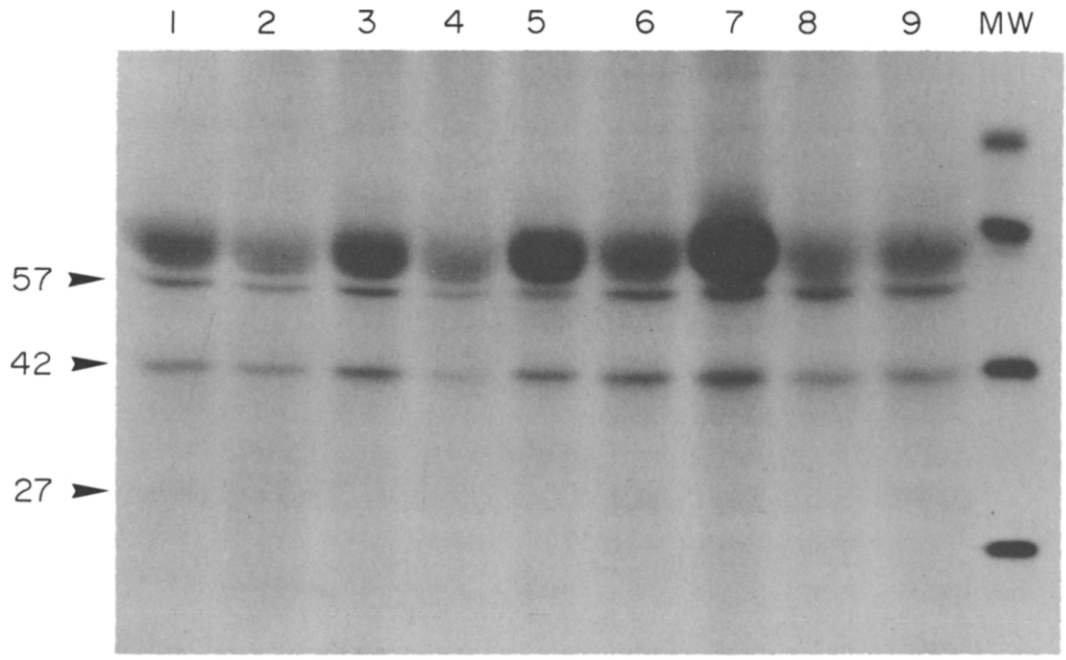

Fig. 1. Autoradiogram of chemical cross-linking of [^{125}I]lanreotide to membrane-enriched pellets isolated from nine breast tumours. Molecular weights of the specific complexes are determined in comparison to the migration of protein markers (MW).

results from two other studies and reveal: (i) 15% of the tumours were positive in the study of Foekens *et al.* [14]. In this study, the presence of somatostatin receptors was determined by autoradiography in cryostat section and was considered as a marker of good prognosis. (ii) 75% of the tumours had receptors for labelled octreotide in the study of Lamberts *et al.* [8]. The somatostatin receptor status was determined by *in vivo* scintigraphy. The difference in results of these three studies makes it uncertain if the somatostatin receptor is a marker of good prognosis for breast tumour. In addition to the presence, the quality and the quantity of somatostatin receptors must be analysed to ensure the pronostic value of this specific parameter.

Moreover, the present results showed that lanreotide binding sites were present in breast adenocarcinomas: (1) with or without oestrogen or progesterone receptors, (2) from menopausal or non-menopausal patients and (3) from all female patients and from 2 male patients. The presence of the various types of lanreotide receptors was not related to the presence of oestrogen or progesterone receptors. This situation is different to that observed in the rat anterior pituitary, where only one of the two characterised subtypes is exclusively expressed after 17-β oestradiol treatment [17, 18].

In conclusion, the antiproliferative activity of lanreotide demonstrated *in vivo* as well as *in vitro* in several mammary cell models [3, 4, 19] and this high percentage of breast tumours which are positive for receptors supports the use of lanreotide in clinical trials. Studies are planned on the molecular heterogeneity of the receptors for somatostatin or its analogues which could lead to a better understanding of the role of each subtype in the control of breast cell proliferation.

1. Brazeau P, Vale W, Burgas R, *et al.* Hypothalamic polypeptides that inhibit the secretion of immunoreactive pituitary growth hormone. *Science* 1973, **179**, 77–79.
2. Thomas F, Parmar A, Prévost G, Kuhn JM, Moreau J, Bogden A. Les analogues de la somatostatine en cancérologie. *Bull Cancer* 1991, **78**, 693–707.
3. Prévost G, Lanson M, Thomas F, *et al.* Molecular heterogeneity of somatostatin analog BIM-23014C receptors in human breast carcinoma cells using the chemical cross-linking assay. *Cancer Res* 1992, **52**, 843–850.
4. Prévost G, Foehrlé E, Thomas F, *et al.* Growth of human breast cancer cells inhibited by the somatostatin analog BIM-23014C (Somatuline). *Endocrinology* 1991, **128**, 323–329.
5. Taylor J, Bogden A, Moreau J, Coy D. *In vitro* and *in vivo* inhibition of human small cell lung carcinoma (NCI-H69) growth by a somatostatin analogue. *Biochem Biophys Res Commun* 1988, **153**, 81–86.
6. Liebow C, Reilly C, Serrano M, Schally A. Somatostatin analogues inhibit growth of pancreatic cancer by stimulating tyrosine phosphatase. *Proc Natl Acad Sci USA* 1989, **86**, 2003–2007.
7. Bogden A, Taylor J, Moreau J, Coy D. Treatment of R-3327 prostate tumors with a somatostatin analogue (somatuline) as adjuvant therapy following surgical castration. *Cancer Res* 1990, **50**, 2646–2650.
8. Lamberts S, Krenning E, Reubi J. The role of somatostatin and its analogs in the diagnosis and treatments of tumors. *Endocrine Rev* 1991, **12**, 450–481.
9. Murthy K, Srikant C, Patel Y. Evidence for multiple protein constituents of the somatostatin receptor in pituitary Tumour cells: affinity cross-linking and molecular characterization. *Endocrinology* 1989, **125**, 948–956.
10. Heiman M, Murphy W, Coy D. Differential binding of somatostatin agonists to brain and adenohypophysis. *Neuroendocrinology* 1987, **45**, 429–436.
11. Yamada Y, Post S, Wang K, Tager H, Bell G, Seino S. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney. *Proc Natl Acad Sci USA* 1992, **89**, 251–255.
12. Moreau S, Murphy W, Coy D. Comparison of somatuline (BIM-23014) and somatostatin on endocrine and exocrine activities in the rat. *Drug Dev Res* 1991, **22**, 79–93.
13. Fekete M, Wittliff A, Schally A. Characteristics and distribution of receptors for (D-Trp6)-luteinizing releasing hormone, somatostatin, epidermal growth factor, and sex steroids in 500 biopsy samples of human breast cancer. *J Clin Lab Anal* 1989, **3**, 137–147.
14. Foekens J, Portengen H, van Putten W, *et al.* Prognostic value of receptors for IGF, somatostatin, and EGF in human breast cancer. *Cancer Res* 1989, **49**, 7002–7009.
15. Reubi J, Waser B, Foekens J, Klijn J, Lambert S, Laissue J. Somatostatin receptor incidence and distribution in breast cancer using receptor autoradiography: relationship to EGF receptors. *Int J Cancer* 1990, **46**, 416–420.
16. Srikant C, Murthy K, Patel Y. Tissue specific distribution of cross-linked somatostatin receptor proteins in the rat. *Biochem J* 1992, **282**, 339–344.
17. Kimura N, Hayafuji C, Kimura N. Characterization of 17-β oestradiol dependent and independent somatostatin receptor subtypes in rat anterior pituitary. *J Biol Chem* 1989, **264**, 7033–7040.
18. Kimura N, Hayafuji C, Konagaya H, Takahashi K. 17 β-estradiol induces somatostatin (SRIF) inhibition of prolactin release and regulates SRIF receptors in rat anterior pituitary cells. *Endocrinology* 1986, **119**, 1028–1036.
19. Thomas F, Prévost G, Bogden A, Moreau J. Somatuline, a somatostatin analog, inhibits the growth of human and rat breast cancer cell lines *in vivo*. In Fiebig HS, Berger D, eds. *Immunodeficient Mice in Oncology*, Basel, Karger, 1992.

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