An accumulation of insulin-like growth factor I (IGF-I) in human myometrium and uterine leiomyomas in various stages of tumour growth

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ABSTRACT. It is commonly thought that uterine leiomyomas result from hyperstimulation of myometrium by ovarian hormones. Some observations suggest that cytokines and growth factors are intermediate elements through which the ovarian hormones may exert their growth-stimulatory effects on leiomyomas. Human myometrium and uterine leiomyomas of various weights were homogenised and extracted with 1M acetic acid or with 0.05M Tris/HCl, pH 7.6. The extracts were assayed for IGF-I using the ELISA technique. It was found that 0.05 M Tris/HCl extracts contained several times more IGF-I than the 1M acetic acid extracts. Nanogram amounts of IGF-I were found in both control myometrium and in leiomyomas. It was found that the amounts of IGF-I extracted from leiomyomas were distinctly higher in comparison to control myometrium and they increased as a function of tumour growth. Polyacrylamide gel electrophoresis, followed by Western immunoblotting, demonstrated that IGF-I in acidic and alkaline extracts exists as stable complexes, probably with extracellular matrix components. No free IGF-I was detected. Furthermore, it was found that some components of both the acidic and alkaline extracts were able to bind exogenous ^{125}I-labeled IGF-I. It is suggested that IGF-I plays an important role both in myometrium biology and in the growth of uterine leiomyomas.

Keywords: IGF-I, human myometrium, uterine leiomyoma, extracellular matrix, IGF- binding

INTRODUCTION

Neoplastic tumours are composed of two discrete compartments: tumour cells and stromal connective tissue. The latter constitutes a significant proportion of the neoplastic mass and provides the infrastructure that the tumour cells require for growth, gas exchange, and waste disposal. Glycosaminoglycans (GAGs) are intrinsic constituents of the extracellular environment associated with a proliferating neoplasm. They are present at the cell surface of most, if not all, tumour cells and in the cell membranes. Most of them (except hyaluronic acid) are bound to protein cores, forming proteoglycans (PGs). They influence the surface charge of plasma membranes, the movement of molecules across tissues, the structure of biological filters, and the migration of cells along defined routes. PGs and GAGs modulate biological activities of tumour cells. They may also serve as a storage site to concentrate and stabilize growth factors in the vicinity of tumour cells. Some of them modify the binding of tumour cells to extracellular matrix components and affect the secretion of hydrolytic enzymes, e.g. proteinases, type IV collagenases and heparitinases, which degrade the extracellular matrix (ECM). Others may modulate tumour metastasis and angiogenesis. In general, underexpression of PGs or decreased GAGs-biosynthesis may inhibit tumour growth [1-3].

It is commonly thought that uterine leiomyomas are the effect of hyperstimulation of myometrium by ovarian hormones. Sozen and Arici [3] suggest that cytokines and growth factors are intermediate elements through which the ovarian hormones may exert their growth-stimulatory effects on leiomyomas Estrogen and progesterone may regulate gene expression of these cytokines and growth factors, which in turn, modify the transcription of other genes. This abnormal production of cytokines and growth factors may result in increased cell proliferation, cellular hypertrophy, accumulation of extracellular matrix, or a combination of these phenomena.

A large number of growth factors have been found to associate with extracellular matrix proteins or with heparan sulphate. Rapid and localised changes in the activity of these factors can be induced by hydrolytic release from matrix storage and/or by activation of latent forms. These growth factors, in turn, may control cell proliferation and differentiation, as well as synthesis and remodelling of extracellular matrix [4]. It is well known that the biosynthesis of ECM components is enhanced by several peptide growth factors, mainly IGF [5], FGF [6] and TGF-β [7].

Several peptide growth factors (TGF-β, EGF, IGF-I, IGF-II, bFGF) have been detected in uterine leiomyoma (for review see [3]). It was found in our preliminary studies and reported by other authors [8, 9], that IGF-I is an abundant growth factor in these tumours.
This report compares the amounts of extractable IGF-I in normal human myometrium and leiomyomas, in consecutive stages of the tumour's growth. Furthermore, the binding of IGF-I by tissue (probably extracellular matrix) components was compared.

PATIENTS AND METHODS

The protocol of this study was accepted by the Committee for Ethics and Supervision on Human and Animal Research of the Medical Academy of Białystok.

Tissue material

Tissues were provided by Professor Stefan Jaworski from the Department of Gynaecology, Medical Academy of Bialystok. Studies were performed on leiomyomas extirpated during surgery. The material was taken from 24 patients. Small tumours weighing less than 10 g (taken from 12 patients, age 41 ± 4) and large tumours weighing more than 100 g (taken from 12 patients, age 44 ± 5) were compared. In each case, the diagnosis was confirmed by histopathological examination of the excised tumour. Control myometrium was taken from 10 patients (age 50 ± 4) undergoing uterus excision because of other clinical indications (ovarian cyst, cervical carcinoma, endometrial polyp). Tissue samples were washed with 0.9% NaCl and stored at -70 ^{o}C, until assay.

Extract preparation

Tissue homogenates (10% w/v) were prepared in 1M acetic acid or in 0.05M Tris-HCl, pH 7.6 using a knife homogeniser (20 000 RPM, 3x 30 sec, 0 ^{o}C). The homogenates were submitted to ultrasonification (20 kHz, 3 x 15 sec, 0 ^{o}C) followed by centrifugation at 10,000 x g for 30 minutes at 4 ^{o}C. The supernatants (tissue extracts) were collected and used for growth factor assay, for Western blot analysis, and for the assay of IGF-I binding.

IGF-I assay

The commercial ELISA kit used for the assay of IGF-I was provided by R&D Systems Inc, Minneapolis, USA (Catalogue number DG 100). The assays of IGF-I in tissue extracts were carried out in duplicate according to the instructions provided by the manufacturer. The amounts of growth factor were expressed per gram of wet tissue.

Sodium dodecyl sulphate / polyacrylamide gel electrophoresis (SDS/PAGE)

Slab SDS/PAGE was performed according to the method of Laemmli [10] in 10% polyacrylamide gel. The extract samples were dissolved in sample buffer (0.0625M Tris/HCI, pH 6.8, containing 20% glycerol and 5% SDS), and 20 µL of each were applied to the gel. The following Bio-Rad molecular mass standards were used: 112.0 kDa, 81.0 kDa, 49.9 kDa, 36.2 kDa, 29.9 kDa and 21.3 kDa. After SDS/PAGE, the gels were allowed to equilibrate in a mixture of 0.025M Tris and 0.2M glycine in 20% (v/v) methanol for five min. The protein was transferred to 0.2 µm pore-sized nitrocellulose, at 100 mA for 1 h using a Sigma-Aldrich SV 20-SDB electrophoresis unit. Nitrocellulose was blocked with 3% IGEPAL CA-630 in TBS for 30 min, then 1% bovine serum albumin (BSA) in TBS for 2 h and finally 0.1% Tween 20 in TBS for 10 minutes at room temperature, as described by Hosenlopp *et al.* [11].

Western blot analysis

After SDS-PAGE, the protein was transferred to nitrocellulose using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with anti-IGF-I monoclonal antibody (R&D, Catalogue number MAB 291), at a concentration of 1:1,000 in 5% dried milk in TBS-T (0.02M Tris-HCl buffer, pH 7.4, containing 0.15M NaCl and 0.05% Tween 20) for 1 hour, followed by alkaline phosphatase-conjugated secondary antibodies against mouse Fc IgG, added at a concentration of 1:7,500 in TBS-T. Incubation was continued for 30 minutes with slow shaking. The nitrocellulose was washed with TBS-T (5 times for five minutes) and submitted to Sigma-Fast BCIP/NBT reagent.

Binding of exogenous IGF-I

The proteins contained in tissue extracts and separated by SDS/PAGE were transferred onto a nitrocellulose membrane and submitted to the ligand binding procedure [11] with radioactive ^{125}I-IGF-I (ICN Biomedicals, INC, USA).

Statistical analysis

Mean values from 10 assays performed on control myometrium and 12 assays performed on leiomyomas ± standard deviations (S.D.) were calculated. The results were submitted to statistical analysis using Student' *t*-test, accepting $p < 0.05$, as significant.

RESULTS

It is apparent from *figure 1* that the extractability of IGF-I depends on the type of extracting solvent. A small amount of IGF-I was extracted by 1 M acetic acid with much more solubilised in 0.05M Tris/HCl, pH 7.6. The small tumours contained three times more, and the large tumours contained more than four times the IGF-I extracted from the control myometrium.
SDS/PAGE separated those proteins reacting with anti-IGF-I antibody into several bands. The electrophoretograms submitted to Western blotting show the positions of IGF-I. As can be seen in *figure 2A* (lanes 1, 2, 3), the acidic extracts did not contain bands characteristic of free IGF-I. Only some weak bands, which reacted with anti-IGF-I antibody, were detected, particularly in the extracts from the large tumours (lane 3).
It is apparent from *figure 2B*, that Tris/HCl (pH 7.6) extracts from control myometrium (lane 1) and from small (lane 2) and large (lane 3) leiomyomas did not contain free IGF-I. However, in contrast to acidic extracts, several distinct bands of material reacting with anti-IGF-I anti-

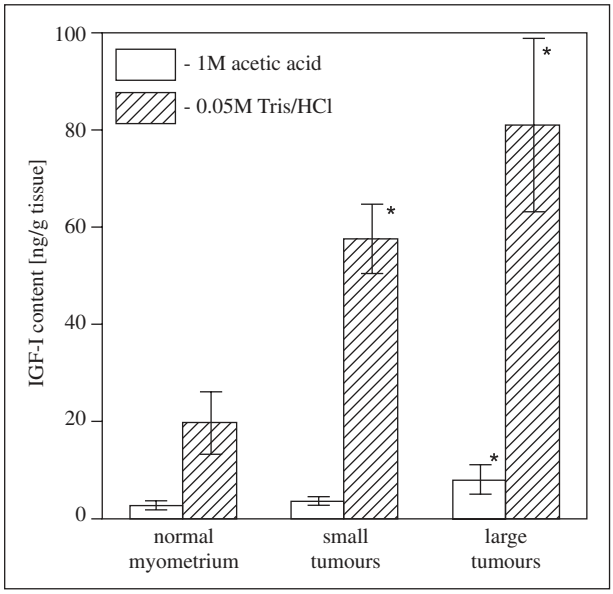

Figure 1
The amounts of IGF-I in the extracts of control myometrium and in leiomyomas of various weights.

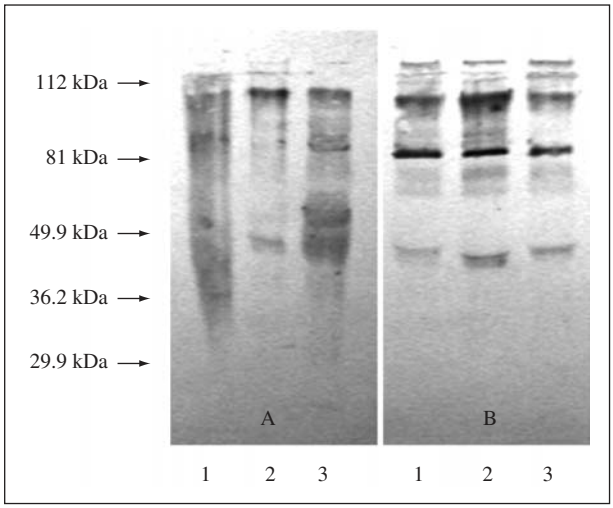

Figure 2
Western immunoblot analysis for IGF-I in the extracts of control myometrium and tumours of various weights. A – 1M acetic acid extracts. B – 0.05M Tris/HCl, pH 7.6 extracts. Lane 1 - control myometrium, lane 2 - small tumours, lane 3 - large tumours.

body were detected; the main one having a molecular weight of about 81 kDa. Similar immunoblots have been obtained in the case of control myometrium, as well as in small and large leiomyomas.

It can be seen *(figure 3A)* (lane 1) that the acetic acid extracts of control myometrium contain some constituents that are able to bind exogenous ^{125}I-IGF-I. One ^{125}I-IGF-I -binding band is apparent at a position corresponding to more than 100 kDa, with two other bands corresponding to a molecular weight of 40-45 kDa. Small leiomyomas (lane 2) did not demonstrate the 100 kDa band whereas the 40-45 kDa bands are distinctly visible. In contrast, the large tumours (lane 3) demonstrated an intense 100 kDa band as well as those corresponding to 40-45 kDa.

The Tris/HCl extracts demonstrated a quite different ^{125}I-IGF-I-binding pattern. Both the control myometrium

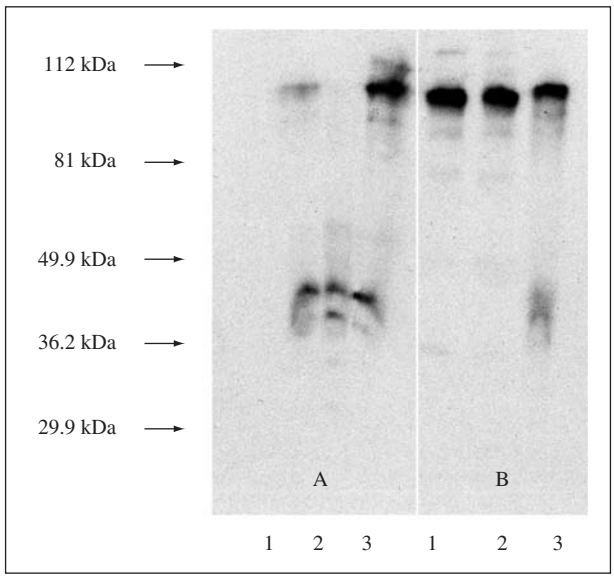

Figure 3
The SDS/PAGE pattern of ^{125}I-IGF-I - binding to components in the extracts of control myometrium and tumours of various weights. A – 1M acetic acid extracts. B – 0.05M Tris/HCl, pH 7.6 extracts. Lane 1 - control myometrium, lane 2 - small tumours, lane 3 - large tumours.

(lane 1) and small tumours (lane 2) contained an homogenous, high molecular weight (100 kDa) component, which strongly binds radioactive IGF-I. The large tumours (lane 3) , also contained the high molecular weight component binding exogenous IGF-I, but in addition showed another IGF-binding substance with a molecular weight of 40-45 kDa.

DISCUSSION

The growth factors exert their regulating roles on various cells by the action on specific receptors. These may be present on the surface of the same cell, which produces the growth factor (autocrine action). Alternatively, the growth factors may work on other cells, which are not themselves producers. In most cases, the target cells lie close to the producer cells (paracrine regulation). In some cases, the target cells may also occur in distant parts of the body, giving rise to a type of regulation analogous to the mode of action of polypeptide hormones (endocrine regulation) [12, 13]. Most of the growth factors investigated exert their regulatory effects by autocrine or paracrine mechanisms. IGF-I is the only peptide growth factor, which apart from its autocrine and paracrine actions, may be released in significant amounts into the bloodstream and transported to target cells located in other organs [12, 13].

It is apparent from the data presented that both human myometrium and leiomyomas contain large amounts (nanogram quantities) of extractable IGF-I. These are distinctly higher in comparison to those of other peptide growth factors, which are extracted at picogram levels, both from these and other tissues [14]. This observation suggests that IGF-I plays a special role in the biology of both normal myometrium as well as in tumour growth.

It is of interest that the extractability of IGF-I from myometrium and leiomyomas is distinctly greater in 0.05M Tris/HCl than in 1M acetic acid, but in no case was free IGF-I detected. Some IGF-I extracted from myometrium

and uterine leiomyomas hardly penetrated the polyacrylamide gel during SDS/PAGE, probably because it forms high molecular weight complexes with extracellular matrix components [4]. Such complexes are very stable. They do not dissociate in denaturing conditions despite the action of SDS and a high temperature (100 °C). It seems that the mechanism of binding of IGF-I, both in myometrium and in leiomyomas, may be different from that in other tissues. Such stable complexes were not detected in other organs. It is known that most plasma IGFs (80-90%) associate with binding-protein-3 (IGF-BP-3). Such a complex readily dissociates in 1 M acetic acid solution. It therefore seems possible that myometrial cells bind IGF-I through a binding component(s) different from IGF-BPs, which exist in plasma and other tissues. It is of special interest that exogenous ^{125}I-IGF-I strongly binds to a high molecular weight component (about100 kDa), as reported for some other peptide growth factors [4].

It is known that some growth factor: ECM interactions involve the binding of such factors (e.g. FGF, EGF) to glycosaminoglycan chains of heparin or to heparan sulphate proteoglycans [4]. Syndecan-2, a transmembrane heparan sulphate proteoglycan binds TGF-β through its core protein [15]. Latent TGF-β–binding proteins are components of the extracellular matrix, structurally related to fibrillin, which are responsible for storage of latent TGF-β in the ECM [16-18]. PDGF binds to secreted protein, which is acidic and rich in cysteine (SPARC), and is a pericellular matrix protein ubiquitously expressed during development, its expression being high in tissues undergoing remodelling or repair [4]. EGF binds to decorin, a small, leucine-rich proteoglycan [19].

IGFs associate with several extracellular binding proteins (IGF-BPs) that regulate the activity of IGFs in tissues [20]. IGF-BP-5 was described as a component of fibroblast ECM, and matrix localisation of IGFs could be due to IGFBP-5 binding [21]. It also binds to types III and IV collagen, as well as to laminin and fibronectin. Such a bound form of IGF-BP-5 is protected from degradation. Increasing salt concentrations inhibited the binding of IGFBP-5 to ECM and accelerated the release of IGFBP-5 from ECM, suggesting an ionic basis for this interaction. The binding of IGF-BP-5 to ECM resulted in a seven-fold decrease in its affinity for IGF-I compared to IGF-BP-5 in solution. This suggests that ECM-bound IGF-BP-5 may have a specialised role in localising IGF to ECM and in mediating its actions. The high affinity of IGF-BP-5 for IGF in solution would allow IGF to remain bound during intercellular transport. The low affinity of ECM-bound IGF-BP-5 for IGF could facilitate delivery of IGF to cell surface receptors. Furthermore, if it is bound to IGFBP-5, IGF is protected from degradation. The high affinity of IGF-BP-5 for IGF during transport, followed by a lowering of its affinity after matrix localisation near IGF cell receptors, could potentiate the biological effects of IGF-I [21].

It is well known that biosynthesis of extracellular matrix components is enhanced by several peptide growth factors, mainly IGF [8], FGF [6] and TGF-β [7]. As we reported in a previous paper [22], leiomyomas contain distinctly more collagen and proteoglycans in comparison to control myometrium. It is apparent from our study that leiomyomas accumulate relatively large amounts of IGF-I. The action of a protease and/or a glycosidase may release and activate the stored IGF-I and generate rapid and highly localised signals [4]. Overproduction and accumulation of IGF-I may be a cause of hyperstimulation of myometrial cell to evoke hyperplasia and tumour growth. Furthermore, these signals may stimulate the leiomyoma cells to produce large amounts of ECM components.

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