Analysis of the relationship between cancer procoagulant activity and PCNA and Ki-67 expression in cases of common and cellular uterine leiomyomas

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Summary

Purpose: Histological subtypes of uterine leiomyomas may substantially differ in their cellular biology, including the intensity of synthesis of cancer markers and expression of cell proliferation markers. The present investigation aimed to determine the activity of cancer procoagulant (CP) in subtypes of leiomyomas, including cellular leiomyomas, and to verify whether these activities correlate with immunoexpression of cell proliferation markers: the proliferating cell nuclear antigen (PCNA) and Ki-67.

Materials and Methods: Preoperative peripheral venous blood and postoperative tissue material were obtained from 24 women operated on in a tertiary referral academic department. The activity of CP in serum was measured with the use of a coagulative method according to Gordon and Benson, and in tissue homogenates with the use of a spectrophotometric method according to Colucci *et al.* The control serum values were obtained from 20 healthy women without any gynecological disease, and the control solid tissue values from histologically confirmed postoperative normal reproductive tissues obtained from six patients. PCNA and Ki-67 expression were determined immunohistochemically using monoclonal antibodies.

Results: Both the tissue and serum activity for CP was considerably higher for common leiomyomas and cellular leiomyomas than for control tissues, but did not differ significantly between the leiomyoma subtypes. Intratumor CP activity significantly correlated with PCNA expression but not with Ki-67 expression.

Conclusions: Cellular leiomyomas do not differ substantially in the serum and intratumor CP activity from common leiomyomas. There is a relationship of intratumor CP activity with PCNA expression, a finding which requires further investigation.

Key words: Cancer procoagulant (CP); Cellular leiomyoma; Common leiomyoma; Ki-67; PCNA.

Introduction

Cancer procoagulant (CP) is a sulfhydryl proteinase thought to be primarily synthesized and expressed by malignant neoplastic cells and fetal membranes cells, while its activity is low in the blood serum of healthy subjects [1, 2]. The enzyme is capable of triggering the coagulation cascade without the activation of coagulation factor VII and via direct activation of coagulation factor X [3]. Consequently, much attention has been directed towards its role as a procoagulant factor in thrombosis-embolism in the course of malignant neoplastic disease [4, 5]. However, it has been recently demonstrated that uterine leiomyomas, representing benign genital neoplasia, synthesize CP like malignant tumors, only to a lesser degree [6].

Uterine leiomyomas, tumors of myometrial origin, are the most common neoplasms of the uterus and occur in 20-30% of subjects over 30 years of age [7]. Despite their high prevalence, the pathophysiology of these tumors is poorly understood. Their subtypes differ in appearance in imaging techniques [8, 9]. Electron microscopic studies revealed that cellular leiomyomas represent a particular variety of the common leiomyoma [10]. Consequently, histological subtypes of uterine leiomyomas may substantially differ in their cellular biology, including the intensity of synthesis of cancer markers and expression of markers of cell proliferation. An important piece of information in this field was provided by Dixon *et al.* who found that proliferative activity is relatively low, variable for individual tumors of the same patient and independent of tumor size, as evaluated by the expression of the proliferating cell nuclear antigen (PCNA) and Ki-67 [11]. In women of reproductive age and in postmenopausal women, the expression of PCNA significantly exceeds the expression of Ki-67 in these tumors [12].

The aim of the present investigation was to determine the activity of CP in the serum and the tissues of women with an array of histological subtypes of uterine leiomyomas. Specifically, it was considered of interest to examine whether cellular leiomyomas are different from common leiomyomas in terms of their CP activity. Additionally, this activity was correlated with expression of PCNA and Ki-67.

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Patients and Methods

Patients and Sample Collection

The study was performed in agreement with the provisions of the Declaration of Helsinki and approved in advance by the Institutional Review Board, Medical University of Bialystok. All subjects gave their informed consent for participation in the study.

The tissue material consisted of postoperative specimens of uterine leiomyomas and portions of macroscopically normal uterine muscular tissues, all subject for histopathological verification. The indication for surgery was the presence of uterine myoma at pelvic examination, confirmed by ultrasound examination. Cellular leiomyomas were identified in six women aged 49 to 67 years (mean 53.8 ± 7.4 years), and leiomyomas in 18 women of 29 to 55 years (mean 44.9 ± 6.0 years). A case of an angioleiomyoma detected in a 43-year-old woman, a case of lipoleiomyoma in a 77-year-old woman, two cases of *leiomyoma hyalinisans* in women aged 38 and 51 years, and a case of leiomyosarcoma in a 66-year-old woman were also included for comparison.

Normal uterine muscular specimens were obtained from six women hysterectomized for the following indications: cervical carcinoma *in situ* (n = 2), suspicion of adenomyosis (n = 2), chronic pelvic pain of unknown cause (n = 2). In cases of adenomyosis, the distance from the focus of endometrial tissue to the site where the normal uterine muscle was sampled was above 2 cm, such that both tissues were not in direct proximity. All specimens were subjected to histopathological confirmation of normal uterine muscle at the site of sampling.

Peripheral venous blood was taken from the patients and from 20 healthy female volunteers, referred to as the control group for blood. Blood was aseptically drawn from an antecubital vein into glass tubes without anticoagulant. In the operated women, the sampling was done at the time of preoperative insertion of the venous access (Dispomed SA, Lublin, Poland), thus not being associated with an additional venipuncture, nor pain. The control group were women aged 23 to 61 years (mean 33.75 ± 11.98 years), in good general health. Pre- and perimenopausal women were sampled shortly after cessation of their regular menses. Blood from the control group was derived in an identical manner as above, in the morning after an overnight fast. Thus, the time of blood sampling, circadian phase, and the fasting state were similar in the examined and control groups. Both solid tissue and coagulated blood were kept at (–20 °C) until analyzed, but not more than three weeks.

The clinical exclusion criteria from the study were: history of viral hepatitis, presence of clinical symptoms of hyperbilirubinemia, and history or presence of symptoms of coagulopathy. The histopathological exclusion criteria were: endometrial polyp(s), endometrial hyperplasia, and external endometriosis.

Analytical methods

The activity of CP was measured in 10% saline homogenates of the tissue specimens and in the blood serum, as previously described [6]. Briefly, for tissue CP, we used a chromogenic method according to Colucci *et al.* [13]. This method is specific for the enzyme and is based on the spectrophotometric determination of the absorbance of para-nitroanilin (pNa) at light wavelength of 405 nm. Results could be read from a standard curve ranging from 0 to 100 nmol pNa/ml. Since bilirubinoids show the maximum of their absorbance at a wavelength of 450 nm, the exclusion from the study of patients with hyperbilirubinemia was necessary to minimize the interference of bilirubinoids on the results.

The activity of CP in the serum was measured with the coagulative method according to Gordon and Benson, with the use of a plasma substrate lacking coagulation factor VII [14]. The activity of CP was expressed as coagulation time in seconds.

Interpretation of the results is as follows: the higher the concentration of pNa, the more pronounced the CP activity in the tissue. The shorter the coagulation time, the more pronounced the CP activity in the serum.

Immunohistochemistry

Paraffin-embedded tissue sections of tumors and normal uterine muscle were subjected to immunostaining using the following monoclonal antibodies (Abs): DAKO/PCNA (Clone PC10) No. M0879 and DAKO/Ki-67 (Clone MIB-1) No. M7240, both at 1:100 dilution. All primary Abs were diluted in PBS with 1.5% normal blocking serum. The studies were performed with avidin-biotin-peroxidase complex (ABC Staining System, Santa Cruz Biotechnology, USA). Slides were counterstained with hematoxylin.

The following immunohistochemical controls were performed: positive controls included breast cancers previously documented as positive for PCNA and Ki-67 immunostaining; negative controls included omission of primary Abs.

The evaluation of immunostaining for PCNA and Ki-67 was analyzed in four different representative tissue fields in high power field (magnification 400 x) by light microscopy, and the mean percentage of cells with positive staining was reported as the final result.

Statistical analysis

All data are expressed as means ± one standard deviation. The statistical analysis was performed using the STATISTICA 6.1 G for Windows (StatSoft, Inc., Tulsa, OK, USA) package. Differences between cellular leiomyomas, common leiomyomas and normal uterine muscle groups were analyzed for significance with the Mann-Whitney U test, except for age differences which were analyzed with the Kruskal-Wallis one-way analysis of variance. Correlations were assessed with Spearman's coefficient for variables of distribution different from normal distribution; $p < 0.05$ was considered statistically significant.

Results

Healthy women in the control group were younger than leiomyoma patients ($p = 0.0020$) or cellular leiomyoma patients ($p = 0.0031$), however, this fact had little or no impact on the comparison of CP. When subdivided, pre- and perimenopausal controls (n = 17), aged 21 to 43 years (mean 29.94 ± 8.02 years) demonstrated a serum CP activity of 294.59 ± 27.38 s, the enzyme's level representative for the follicular phase of the cycle. Three

Table 1. — *Cancer procoagulant (CP) activity in histological subtypes of uterine leiomyomas and in a case of leiomyosarcoma. Values are given as means ± SD.*

Anatomopathological finding	CP activity in serum (s)	CP activity in tissue (nmol pNa/ml)
Leiomyoma (n = 18)	189.39 ± 21.17	17.68 ± 3.29
Cellular leiomyoma (n = 6)	173.33 ± 41.51	21.05 ± 5.57
Angioleiomyoma (n = 1)	169	17.25
Lipoleiomyoma (n = 1)	260	16.25
Leiomyoma hyalinisans (n = 2)	152.50 ± 26.16	21.25 ± 3.18
Leiomyosarcoma (n = 1)	108	27.25

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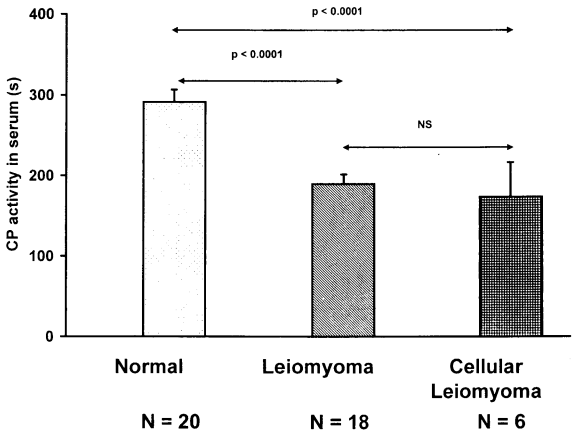

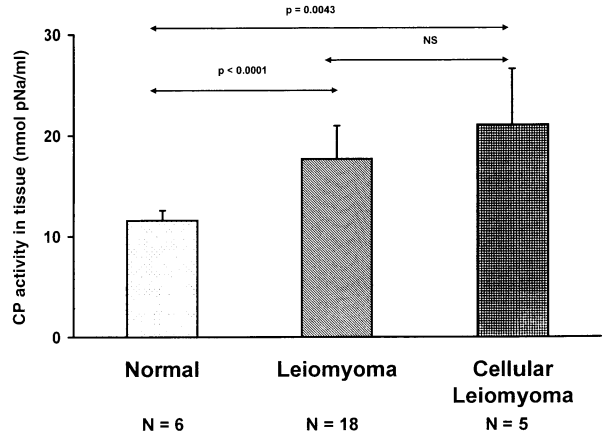

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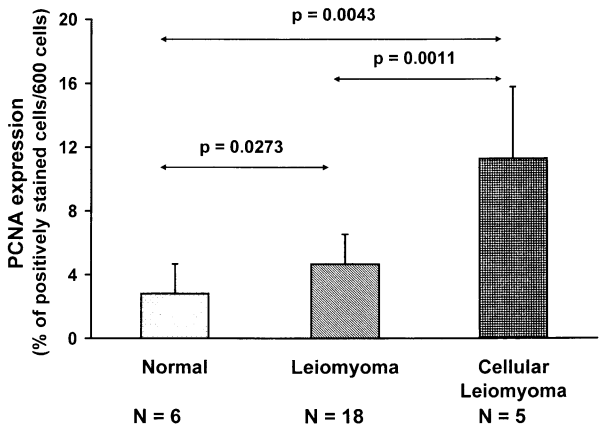

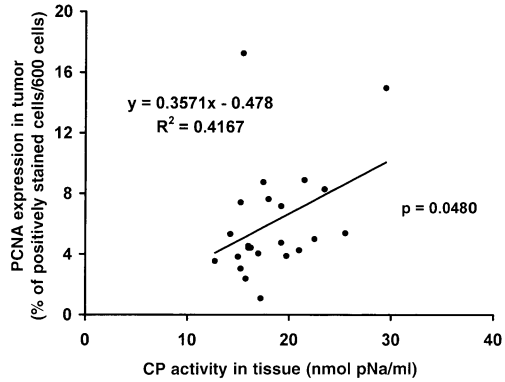

Fig. 4

Figure 1. — Cancer procoagulant (CP) activity in serum of patients with common uterine leiomyomas and cellular leiomyomas. CP activity was determined chronometrically as the coagulation time with the Gordon and Benson method [14]. NS: not significant.
Figure 2. — Cancer procoagulant (CP) activity in tissue. CP activity was determined spectrophotometrically with the Colucci *et al.* method [13]. pNa: para-nitroanilin; NS: not significant.
Figure 3. — Expression of the proliferating cell nuclear antigen (PCNA) in tissue. PCNA was determined immunohistochemically with monoclonal antibody. NS: not significant.
Figure 4. — Correlation of cancer procoagulant (CP) activity with proliferating cell nuclear antigen (PCNA) expression in combined common and cellular leiomyomas. Number of observations: *n = 23*. R: Spearman coefficient.

postmenopausal women, aged 54 to 61 years (mean 55.33 ± 5.13 years), had a serum CP activity of 273.33 ± 15.54 s, a value not significantly different (p = 0.2140) from that found in younger controls.

In all the reference groups for normal blood serum (n = 20), the obtained values of 291.40 ± 26.78 s were similar to those reported for normal subjects in the original method [14], and by our laboratory [6, 15]. These values were highly significantly different from CP activity in serum of patients with both leiomyoma and cellular leiomyoma, indicating the presence of CP in the serum of all affected women at a comparable level (Table 1, Figure 1). However, there was no appreciable difference in this activity between cellular leiomyomas and leiomyomas (p = 0.0769). Within the examined array of tumors, the least pronounced serum CP activity was noted for a case of lipoleiomyoma, and the most pronounced activity for a leiomyosarcoma (Table 1).

The CP activity in the tissues was as follows: for normal uterine muscle it was 11.58 ± 1.00 nmol pNa/ml, while in leiomyoma - 17.68 ± 3.29 nmol pNa/ml and in cellular leiomyoma - 21.05 ± 5.57 nmol pNa/ml. These values were not significantly different (p = 0.1736) within the subtypes of leiomyoma while the values for tumors were significantly different from the reference values (Table 1, Figure 2). For all the examined tumors, the most pronounced tissue CP activity was found for leiomyosarcoma (Table 1).

The expression of Ki-67 antigen was very low in all the three tissue types examined (Figure 3). It was: 0.76 ± 1.10% of positively stained cells/600 cells for normal uterine muscle, 0.59 ± 0.39% of positively stained cells/600 cells for leiomyoma, and 1.61 ± 1.48% of positively stained cells/600 cells for cellular leiomyoma. No significant difference was noted between these values (p = 0.4942 for normal uterine muscle vs leiomyoma; p = 0.3290 for normal uterine muscle vs cellular leiomyoma; p = 0.2268 for leiomyoma vs cellular leiomyoma).

The expression of PCNA antigen was not significantly different from that of Ki-67 for normal uterine muscle ($p = 0.4100$), whereas it was significantly evident for both leiomyoma ($p < 0.0001$) and cellular leiomyoma ($p = 0.0080$). It was: 2.80 ± 1.87% of positively stained cells/600 cells for normal uterine muscle, 4.66 ± 1.87% of positively stained cells/600 cells for leiomyoma, and 11.28 ± 4.51% of positively stained cells/600 cells for cellular leiomyoma. These levels were significantly different from one another (Figure 3).

Further, the possible correlation of intratumor CP activity with Ki-67 and PCNA expression was examined. As expected, for normal uterine muscle, no significant CP versus Ki-67 ($p = 0.0670$) or CP versus PCNA ($p = 0.2580$) correlations were observed. For leiomyomas, the CP versus Ki-67 correlation was not significant ($p = 0.1960$), nor was the CP versus PCNA correlation ($p = 0.2240$). Somewhat similar, for five cases of cellular leiomyomas, neither the CP versus Ki-67 ($p = 0.3340$), nor the CP versus PCNA ($p = 0.9120$) correlation proved to be of any significance. However, when the number of observations was increased by pooling the leiomyoma and cellular leiomyoma data ($n = 23$), there was no relationship of CP activity with Ki-67 expression ($p = 0.8990$), and, in contrast, there was a significant direct correlation of CP activity with PCNA expression (Figure 4). This observation was further supported by the analysis of 25 cases, i.e., when an additional two cases of *leiomyoma hyalinisans* were included. The CP versus Ki-67 correlation was not significant ($p = 0.7940$), whereas the CP versus PCNA correlation was of borderline significance ($p = 0.0630$).

Discussion

The results obtained for CP activity in normal uterine muscle (11.58 ± 1.00 nmol pNa/ml) were slightly lower than those previously obtained for this tissue in another set of samples (13.2 ± 2.2 nmol pNa/ml) where autologous uterine muscle was sampled for reference [6]. These results are similar to the values reported for normal cervical epithelium (12.04 ± 1.05 nmol pNa/ml) and endometrium (12.50 ± 2.11 nmol pNa/ml) [15]. It seems that normal values for genital epithelial and smooth muscle tissues in the Colucci method are approximately 12 nmol pNa/ml.

Although the histogenesis of leiomyomas is suggested to be unicellular in origin [7], their apparently differentiated forms have been distinguished, like cellular and other leiomyomas [10]. Our results indicate that these histologic types do not differ substantially in their serum and intratumor CP activities. However, the activity of CP in homogenates of leiomyomas and in serum of patients with such lesions is higher than both in homogenates of normal tissues and in normal serum. This is the second study to confirm the initial work by Jóźwik *et al.* [6] on the presence of CP activity in benign neoplastic disease.

It is well known that neoplasms of both non-genital and genital localization are associated with the activation of coagulation [4, 5]. There is reputable evidence to buttress that some leiomyomas cause additional procoagulative mechanisms by triggering polycythemia [16, 17]. In agreement with the results of the present study, namely with the increased activity of CP in leiomyomas, one can expect in these clinical conditions the activation of coagulation since CP directly activates coagulation factor X [3]. Accordingly, there may be a predisposition in women with uterine leiomyomas to thromboembolic events via the CP pathway.

Another important aspect of this study was the possibility to evaluate CP activity in leiomyomas in reference to the status of PCNA and Ki-67. PCNA is the auxiliary protein of DNA polymerase delta and epsilon [18]. It is closely related to p53 protein: the PCNA gene is induced by p53, and PCNA protein interacts with a number of p53-controlled proteins [19]. The interplay of PCNA/p53 levels decides whether DNA synthesis, replication, repair or methylation occur [19, 20]. Ki-67 is a labile nonhistone protein of nuclear localization which is present when the cell is in the mitotic cycle, and absent when the cell is in the G0 phase [21]. Immunohistochemical determination of expression of the two proteins is a widely used method for determination of the proliferative potential of tumors. Our study demonstrates very low levels of Ki-67 and relatively low levels of PCNA, as described in two earlier reports on leiomyomas [11, 12] and a recent report on cellular leiomyomas [22]. It further extends this observation by a preliminary finding of a correlation of intratumor CP activity with PCNA expression, but not with Ki-67 expression.

A possible explanation for the CP/PCNA relationship needs to be considered. Since leiomyomas contain receptors for estrogens and progesterone and remain under the profound control of sex hormones [23], it is now appropriate to examine the correlation of CP activity with the status of estrogen and progesterone receptors in leiomyomas. There is a good validation for such line of future research. *In vitro* studies indicate that in cultured leiomyoma cells both estradiol and progesterone increase PCNA expression [24].

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