The Egyptian Journal of Hospital Medicine Vol., 14 : 1- 10 March 2004
I.S.S.N: 12084
1687 - 2002

A Correlative Study of Angiogenesis Extent and Expression of Matrix Metalloproteinase-9 with Upgrading and Myometrial Invasion in Endometrial Endometrioid Carcinoma

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Abstract:

Changes in angiogenesis and expression of extracellular matrix-degrading enzymes have been substantiated during tumor changeover and progression. This study was carried out on 60 retrospective endometrial endometrioid carcinoma (EEC) cases in addition to 15 normal endometrial biopsies as controls. EEC cases were grouped according to both histological grade (G), from G1 to G3, and the depth of myometrial (M) invasion, from M1 to M3. The study investigated all cases immunohistochemically to determine their microvessel number and the expression of matrix metalloproteinase-9 (MMP-9) and showed significantly high counts in EEC as a whole over the control endometria ($P < 0.001$). Moreover counts of the G1 group overlapped those of the control endometra, increased significantly ($P < 0.01$) in the G2 and even more in the G3 group. G3 cases, in particular, displayed most microvessels widely scattered in the tumor tissue, in close association with tumor cells and as winding and arborized tubes, often dilated in microaneurysmatic segments. The counts also increased in M2 and M3 ($P < 0.001$) while those of the M1 group overlapped the counts of control endometria. Expression of MMP-9, evaluated as percentages of positive cases, revealed that the overall EEC cases gave a significant increase ($P < 0.01$) over the normal control endometria. Also, the frequencies of expression were significantly increased with the histologic grade ($P = 0.01$) and with the depth of myometrial invasion ($P = 0.08$). The increases for MMP-9 were more evident on transition from G2 to G3 than from G1 to G2. The relationship to the depth of invasion revealed that the increases for MMP-9 were found at each depth, mostly on transition from M2 to M3. By contrast, only two of the control biopsies (13.5%) expressed few MMP-9. In EEC, MMP-9, as well, was, expressed by the host stromal cells.

These data suggest that angiogenesis and degradation of extracellular matrix occur simultaneously with EEC upgrading and advancing depth of invasion. Also, they suggest that EEC cells and some host stromal cell populations cooperate in the tumor progression.

Key words: Angiogenesis, Matrix metalloproteinase-9, Endometrial neoplasms, Endometrioid carcinoma, Tumor invasion, Tumor progression.

Introduction:

Angiogenesis; the formation of new blood microvessels, is an obligatory event connected with tumor growth invasion and metastasis[1]. Faster growing, highly invasive and metastatic tumors need more vessels[2]. The endothelial cells of microvessel sprouts secrete important paracrine growth factors for tumor cells[3] as well as several extracellular matrix-degrading enzymes which allow spread of the tumor cells into and through the adjacent matrix[4]. The new microvessels permit metastases because an expanding endothelial surface increases opportunities for tumor cells to enter the circulation[5].

The additional events involved in tumor progression comprise the secretion of matrix metalloproteinases (MMPs). The MMP family plays an important role in the proteolysis of various components of

extracellular matrix & it includes type IV collagenases of 72 KDa (MMP-2) and of 92 KDa (MMP-9) that degrade the type IV, V, VII and X collagens as well as fibronectin[4]. Thus, highly invasive tumor cells would be expected to secrete large amounts of these proteolytic enzymes[6].

There is an evidence that angiogenesis is enhanced in keeping with the invasive and metastatic phases of solid tumors including colon[7] , breast[8] and lung[9] carcinomas as well as melanoma[10]. Relationship of this type also hold true for overexpression of MMP-9[11,12]. In endometrioid carcinoma, however, the knowledge is circumstantial. Angiogenesis is associated with tumor changeover[13] and poor histological differe-ntiation; grade 3,[14] and MMP-9, in particular is overexpressed in grade 3 and metastatic tumors[15].

This study, therefore, aims at investigating the angiogenesis extent and expression of MMP-9 in endometrial endometrioid carcinoma (EEC) biopsies and correlating them with the tumor progression as defined by the histologic grades and the depth of myometrial invasion.

Material and Methods:

The study was performed on sixty (60) retrospective cases of endometrial endometrioid carcinoma (EEC) undergoing total abdominal hystrectomy, bilateral saplingo-oophorectomy, peritoneal cytology and pelvic lymph node sampling. Post-operative follow-up (range 16-60 months, median 48 months) consisted of pelvic examination and vaginal cytology every three months for the first two years and every six months thereafter. C.T scan and chest X-ray were performed yearly. The patient's age, the tumor stage & grade and the depth of myometrial (M) invasion as well as the data of post-operative follow-up were obtained from the patient's files. Additional fifteen(15) normal endometrial biopsies used as controls, were taken by dilatation and curettage during the mid-secretory phase, when angiogenesis is low (15), from women (ages 23-44 years), with no apparent endocrine dysfunction, examined at the Department of Obstetrics and Gynecology, AL-Husein and Bab Al-Shaariah University Hospitals. All subjects gave their informed consent for tissue collection. EEC specimens were obtained from the Pathology Department, Al-Azhar University and Kasr El-Aini Hospitals during the period *(1997-1999)*.

Formalin- fixed, paraffin-embedded blocks were sectioned at 5μm thickness. One section was stained with Haematoxylin and Eosin, for routine histopathologic study and confirmation of the diagnosis. Additional two sections were used for immunohist-ochemical study to analyze the microvessel density (MVD) and MMP-9 expression. Murine monoclonal primary antibodies for the endothelial cell marker; factor VIII (MoAb M 616, Dako, Glostup, Danmark) and for MMP-9 (Fuji Chemical Industries, Ltd, Takaoka, Japan) were applied each one on a section. Antigen retrieval was done by microwave heating in citrate solution (Biogenex-Neufahrn, Germany). Secondary anti-mouse antibodies using peroxidase labeled Biotin Streptavidin Complex detect-ion system (Dako, Copenhagen, Denmark) were then applied. Counterstaining was performed using Mayer's Haematoxylin.

Assessment of Angiogenesis

Care was taken to select microvessels e.g. capillaries and small venules, from all the stained vessels. A simultaneous identification, by two investigators, of these microvessels as transversally sectioned tubes, with a single layer of endothelial cells, either with or without a lumen and not exceeding 10μm in diameter was performed. Each identification was agreed upon in turn. A slightly modified plani-metric point-count method[10] was used to count microvessels. Six to eight 160 X fields (0.82mm^2 per field), covering almost the whole section, were analyzed with a 144 point –mesh inserted in the eye piece. The microvessel number was calculated as the total number of the mesh intersection points occupied by transversally sectioned microvessels. Because of the small size of the transversally sectioned microvessels

and the sufficient distance between two adjacent intersection point, one given point could be occupied by only one microvessel. In contrast, both the microvessels transversally sectioned but placed on the inside or on the sides of a given small square of the mesh and those longitudinally or tangentially sectioned, regardless of their position, were not counted. The mean ± 1 standard deviation, median and range were calculated per section and groups of biopsies.

Immunohistochemistry (IHC) of MMP-9:

The slides, prepared as described previously, were also examined under light microscopy by two investigators independently. For the upregulation of MMP-9, existence of more than 25% immunoreactive cells was considered positive.

Statistical analysis:

The significance of changes in microvessel number and MMP-9 expression in the groups of normal endometria and EEC biopsies was assessed by Fisher's exact test and the non parametric Kruskal-Wallis test. A linear regression test was applied to relate the percentages of MMP-9-positive & -negative EEC tissues with the histological grades and the depth of myometrial (M) invasion. All data were analyzed by the Pearson's Chi-square test.
A *p- value* was found to be statistically: Significant if ≤ 0.05; highly significant if ≤ 0.01; very highly significant if ≤ 0.001 & insignificant if > 0.05.

Results:

The EEC histological grades were assessed according to the International Federation of Gynecology and Obstetrics (FIGO) criteria[16] as (i) G1 (well differentiated), with $\geq 95\%$ of glandular and /or papillary structures, including 20 cases, (ii) G2 (moderately differentiated, Fig. 1) with $> 50\%$ of glandular and /or papillary structures, including 18 cases, and (iii) G3 (poorly differentiated, Fig. 2), with $> 50\%$ solid areas, including 22 cases. The depth of myometrial (M) invasion was also classified according to the FIGO criteria[16] as (i) M1 (22 biopsies), (ii) M2 (18 biopsies) and (iii) M3 (20 biopsies) regarding the extension of EEC to the inner third, the middle third and the outer third of myometrium, respectively. There was a significant correlation ($P = 0.05$) between the grades and the depth of invasion. Nine patients (15%) were found to have positive peritoneal cytology (FIGO stage IIIA) and 6 patients (10%) have an involvement of pelvic lymph nodes (FIGO Stage IIIC) (Table 1). The follow-up revealed fourteen recurrences (23%), all, except two, were in the pelvis. Six patients with recurrent tumors died of the disease between 36 and 38 months and the remaining eight were alive and well on a follow-up of 16 –22 months after diagnosis of recurrence.

The number of microvessels in tissues from the normal control endometria and EEC, as a whole and as groups from G1 to G3 and from M1 to M3, were found in Table 2. Comparison between groups revealed statistically significant differences ($P = 0.004$). When the differences between groups were considered, there were higher counts in the overall EEC compared to the normal endometrium ($P = 0.002$). When each grade was compared to the control, no significant difference was observed in the G1 biopsies whereas both G2 and G3 biopsies disclosed significantly higher counts ($P = 0.06$ and $P = 0.003$, respectively). The intergrade comparison revealed that the counts increased in the function of upgrading, higher counts in G2 versus G1 ($P = 0.01$) and higher in G3 versus G2 ($P = 0.05$). Regarding the depth of myometrial (M) invasion, the count of M1 group overlapped that of the controls but increased significantly ($P = 0.007$) in M2 group and persisted in M3 group. Histologically, microvessels were found in all tissues as endothelial cells, single or clustered in nests or tubes, either with or without a lumen, not exceeding $10\mu m$. In control and EEC G1 (Fig. 3) biopsies, microvessels were mainly confined near the epithelial cells and only scarcely found within the spared inflammatory tissue. Conversely, G2 and, more copiously. in G3

biopsies, they were spread throughout the tumor tissue. In G2 (Fig. 4) and, more evidently, in G3 biopsies, the microvessels could usually be found as tortuous, arborized and anastomosed tubes, some of which displayed microaneurysmatic dilatations. The pictures of this type were rarely encountered in control as well as in G1 biopsies.

The MMP-9 was expressed with a cytoplasmic pattern and in all EEC grades, it was not expressed by the whole tumor cell population but by single cells or cell nests distributed in the tumor area, which gave a very heterogenous "ground-glass-like picture. Also, it was expressed by large stromal cells. The number and percentages of biopsies expressing MMP-9 were found in Table 3. The overall EEC gave a significant increase (P = 0.06) in the frequency of MMP-9 expression over the normal biopsies. Also, the frequencies were significantly increased with advancing histologic grading (P = 0.01) and with increasing the depth of myometrial invasion (P = 0.08). Increases for MMP-9 were more evident on transition from G2 (Fig. 5) to G3 (Fig. 6) than from G1 to G2. Moreover, the relationship to the depth of invasion revealed that the increases for MMP-9 were found at each depth, mostly on transition from M2 to M3. By contrast, only two out of the fifteen control biopsies (13.5%) expressed few MMP-9.

Table (1): Clinical and histopathological variables of the studied biopsies (n = 60):

*Grade (G)	No; of biopsies	Average age (median, range)	*Stage					* Depth of myometrial (M) invasion		
			IB	IC	IIA	IIB	IIIA-C	M1	M2	M3
G1	20	63 (61,43-81)	8	3	3	3	3	14	3	3
G2	18	65.5 (62,52-86)	8	1	3	2	4	4	7	7
G3	22	70.3 (69,63-82)	2	9	2	1	8	4	8	10

** Grade, stage and depth of myometrial (M) invasion were assessed according to the FIGO criteria [(16)].*

Table (2): Microvessel counts in studied biopsies:

Biopsy	No; of biopsies	No; of microvessels	Per 0.82mm^2
		Means ± S.D.	(Median; range)
Normal endometrium	15	3.7 ± 1.3	(3 ; 1-10)
Endometrioid carcinoma:	60	8.9 ± 6*	(7 ; 1-26)
Histologic Grade 1	20	4.7 ± 2.9	(5.5 ; 1-15)
Grade 2	18	8.7 ± 5.9	(6.5 ; 4-21)
Grade 3	22	13.8 ± 5.1*$^{\bullet}$	(12.5 ; 9-26)
Depth of invasion:			
M1	22	4.7 ± 2.8	(5 ; 1-13)
M2	18	12.2 ± 6$^{\bullet}$	(12 ; 7-18)
M3	20	13.0 ± 4.0	(12 ; 9-26)

S.D. = Standard deviation, * P < 0.001 as compared to normal biopsies

$^{\bullet}$ *P < 0.01 and* *$^{\bullet}$ *P < 0.05 as compared to the preceding group.*

Table (3): Expression of matrix metalloproteinase-9 (MMP-9) by the studied biopsies:

Biopsy		Total No;	MMP-9-positive biopsies	
			No;	%
Normal endometrium		15	2	13.4
Endometriod carcinoma:		60	22	36.6
Histologic	Grade 1*	20	4	20
	Grade 2	18	6	33.3
	Grade 3	22	12	54.5
Depth of myometrial (M) invasion:				
	M1	22	4	18.2
	M2	18	5	27.7
	M3	20	13	65

**Grade & depth of myometrial invasion were assessed according to the FIGO criteria [(16)].*

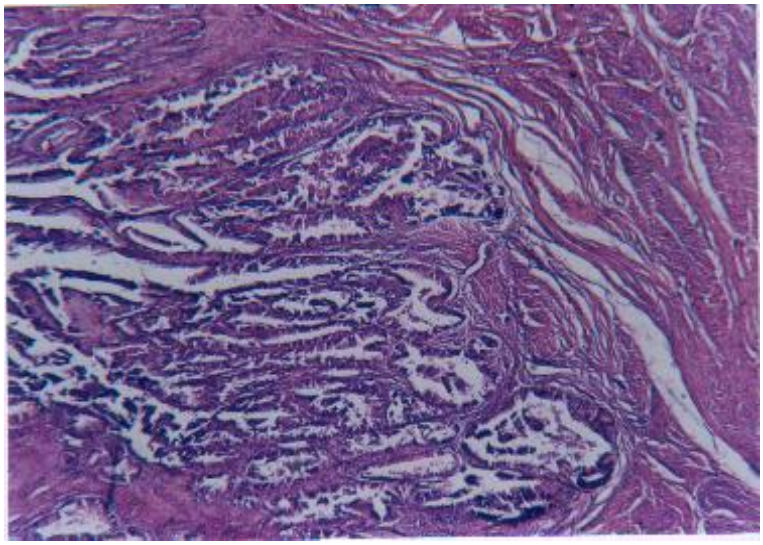

Fig. (1): A case of EEC, grade 2, showing glandular and papillary structures invading the myometrium (Hx & E X 100).

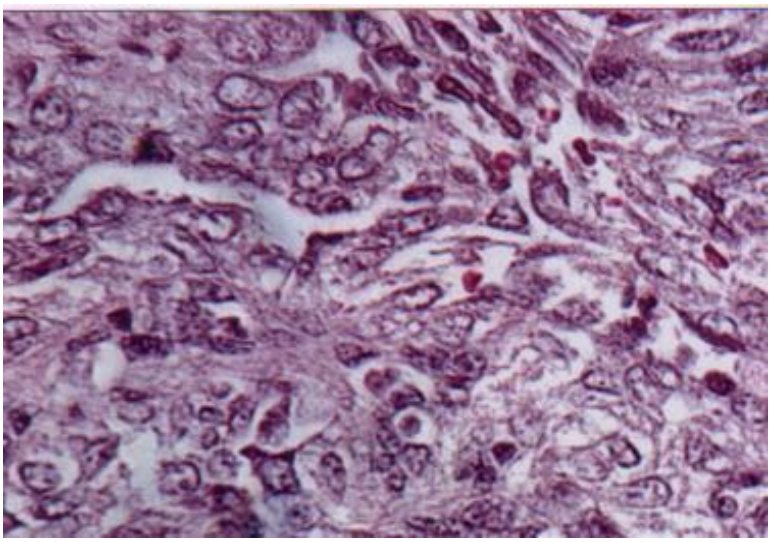

Fig. (2): A case of EEC, grade 3, showing a solid sheet of pleomorphic malignant cells with a rudimentary glandular lumen. (Hx & E X 400).

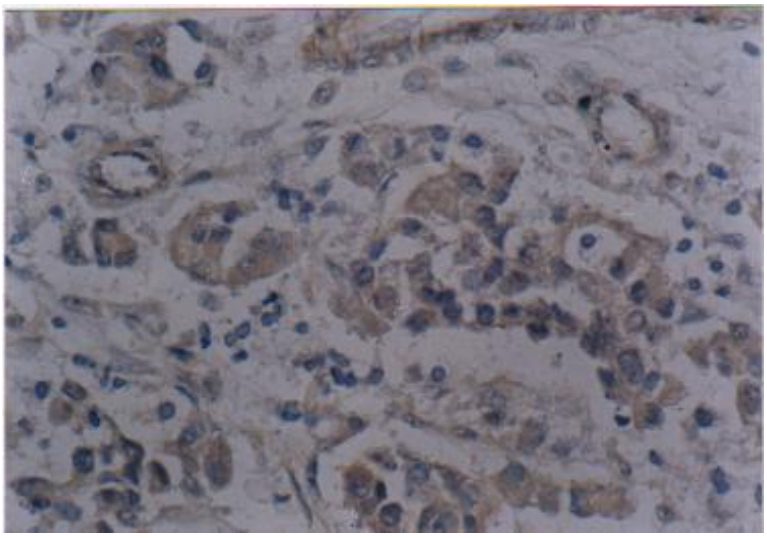

Fig. (3): A case of EEC, grade 1, showing clusters of microvessels confined near the malignant epithelial cells. (Factor VIII X 200).

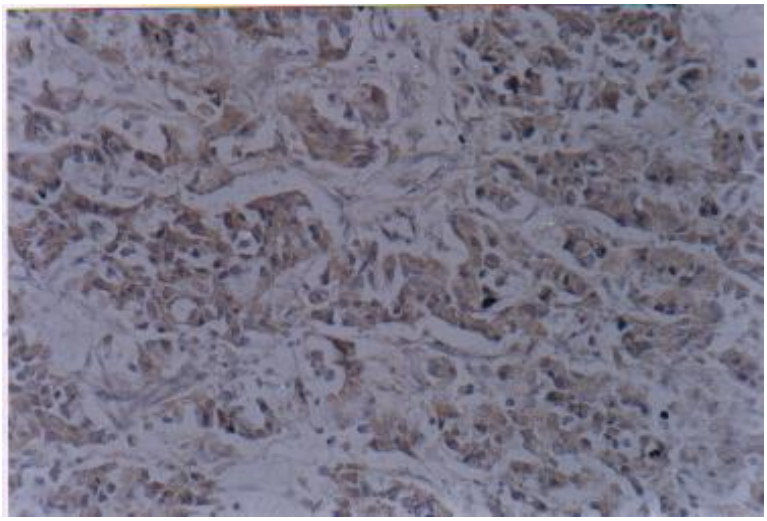

Fig. (4): A case of EEC, grade 2, showing arborized and tortuous microvessels found throughout the tumor tissue. (Factor VIII X 100)

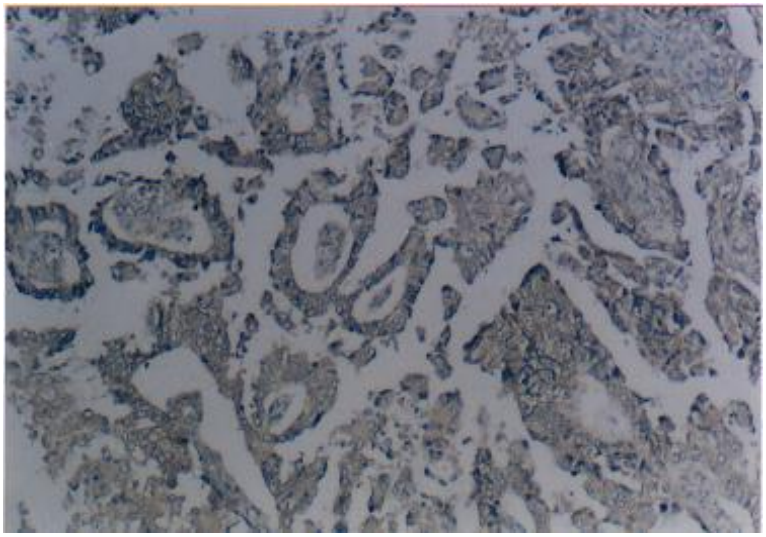

Fig. (5): A case of EEC, grade 2, positive for MMP-9 immunoreactivity (Brownish cytoplasmic immunostaining). (Immunoperoxidase [IP] X 100).

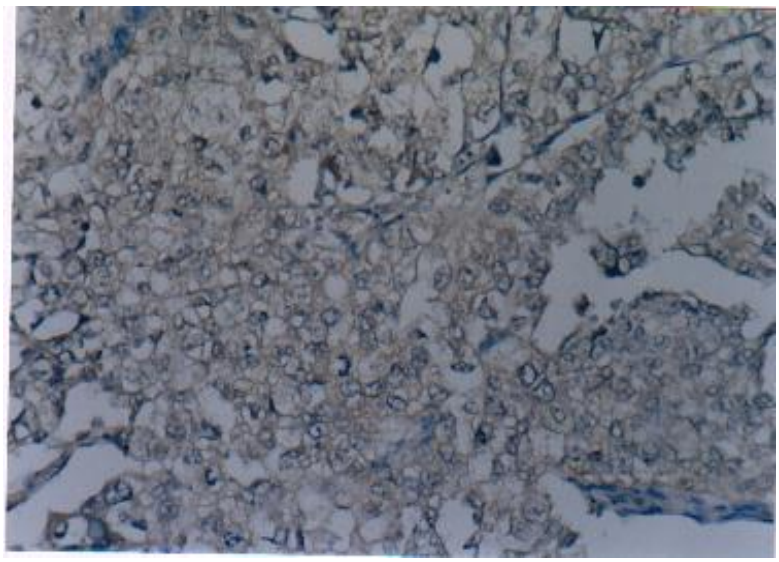

Fig. (6): A case of EEC, grade 3, positive for MMP-9 immunoreactivity. (IP X 200).

Discussion:

Angiogenesis and MMPs are important events in the tumor growth and progression. G1 to G3 offer a clear representation of tumor progression because[16,17]: (a) The tumor growth fraction (S-phase fraction) rises significantly in the transition from G1 to G2 and from G2 to G3; (b) Clinical and morphological evolution from one grade to the next is typical. In particular, MMP-9 was predominantly correlated with the pathological stage and histological grade in EEC which displayed a highly characteristic immunophenotype of the endometrial carcinomas[18].

This study showed that angiogenesis extent, evaluated as microvessel number, and the expression of MMP-9 by the tumor cells were increased simultaneously according to the advancing histological grade (G) and depth of myometrial (M) invasion in EEC. The microvessel counts were low in G1 (overlapping those of normal control group) and increased significantly in G2 ($P < 0.01$) and even more so in G3 ($P< 0.001$). In addition, the counts in M1 group overlapped those of the control one, although being significantly increased in M2 & M3 groups ($P < 0.001$). These results agreed with those of *Wagatsuma et al.*,[14] who found higher counts in G3 versus G1 + G2 and in M2 and M3 versus M1, though normal endometrium was not considered. Although in situ, but not a functional, assessment was performed in this study, it is suggested that new microvessels are induced by EEC cells, whose angiogenic ability is enhanced with advancing grades or with tumor dedifferentiation. Angiogenesis could be stimulated directly or indirectly, after the tumor cells have recruited inflammatory cells (macrophages, mast cells and lymphocytes) stimulating them to secrete their own angiogenic factors[5]. In addition, mast cells, a prominent stromal cell population in the endometrium[19], are well recognized to be involved in the tumor angiogenesis[20] since the tumor cells activate them to produce the angiogenic histamine and tryptase [21] as well as an array of angiogenic cytokines[22]. Neovessels favour the tumor invasion and metastasis[5] which could explain why EEC G3 tumors are more frequently metastasized to pelvic peritoneum, adnexa, vagina and lymph nodes (i.e. found in the stage III), as detected in 8 G3 (36%) versus 4 G2 (22%) and 3 G1 (15%) biopsies in the present study. These figures coincided with those reported by Iurlaro et al., [18]

MMP-9 detected in this work was significantly overexpressed from G1 to G3 biopsies and the increase in frequency of tumors positive for MMP-9 was encountered with advancing grades ($P = 0.01$) as well as with deeper myometrial invasion ($P < 0.01$). The data, suggest that MMP-9 is produced more frequently and greatly as EEC progresses and hence a degradation of the interstitial stroma and subendothelial basement membrane is more intense with

progression. They agreed with those of *Inoue et al.*,[15] who showed that the expression of MMP-9 was associated with vessel invasion, highest grade (G3) and lymph node metastasis and were mimicing as well, those reported by *Iularo et al.*,[18] who could explain the greater dissemination and deepening of endometrial carcinoma cells into the myometrial wall as they undergo the transition from G1 to G3. Together with denser angiogenesis, they could additionally account for easier spreading to lymph nodes and other parenchymal organs with that transition.

In this study, all investigated EEC showed that MMP-9 was also expressed by large stromal cells. This finding was similar to that reported by *Hampton et al* [23] and *Osteen et al*[24] who found that certain stromal cells in EEC, belonging to a subset of macrophages, expressed MMP-9 and they as well, produced additional matrix-degrading enzymes, such as interstitial collagenase, or MMP-1, and stromelysin-1 or MMP-3, when plasma progesterone and estradiol levels decline, thus, overcoming their tissue specific inhibitors (TIMPs) and leading to tissue digestion and breakdown in the last days of the secretory phase and in menstruation[25,26].

In the endometrial carcinoma, several stromal cells, activated by tumor cells, produce MMP-2 and MMP-9, thus participating in the degradation of the extracellular matrix, and enhancing the tumor dissemination[15]. Together with the findings in other solid tumors [27,28], our results suggest that the regulation of extracellular matrix degradation during tumor progression is the result of a concerted action not only of several proteolytic enzyme systems, but also of several cell types, including both malignant and non-malignant cells in the neoplastic stroma.

In conclusion our, data show that angiogenesis and MMP-9 overexpression occur simultaneously during EEC progression. This suggests that there are more chances for these malignant cells to enter the circulation and spread systemically in parallel with progression. The use of antiagiogenic agents[29] and/or tissue inhibitors of metalloproteinases (TIMPs) [26] may be a target for therapy.

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دراسة علاقة حجم توالد الأوعية الدموية الدقيقة واظهار انزيم ميتالوبروتينيز-9 مع التدرج التصاعدى والغزو العضلى لسرطان الجدار المبطن للرحم

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تتجسد التغيرات فى توالد الأوعية الدموية الصغيرة وكذلك فى إظهار الإنزيمات المحللة للنسيج الواقع بين الخلايا أثناء تطور وتقدم الورم وقد أجريت هذه الدراسة على 60 حالة من سرطان الجدار المبطن للرحم بالإضافة الى 15 حالة من الجدار الطبيعى للرحم على سبيل المقارنة. وقد تم دراسة كل من عدد الأوعية الدموية الدقيقة وإظهار انزيم ميتالوبروتينيز-9 بالمناعة الهستوكيميائية و أظهرت الدراسة أن اعداد الأوعية الدموية الدقيقة أعلى فى حالات السرطان منها فى النسيج الطبيعى ، كما أنها بينت وجود تداخل فى الأعداد بين سرطان الدرجة الأولى والنسيج الطبيعى ولوحظ أيضاً ازدياد أعداد هذه الأوعية فى سرطان الدرجة الثانية عنه فى الأولى وفى الدرجة الثالثة عنه فى الثانية بالإضافة الى ذلك فقد زادت اعداد هذه الأوعية فى سرطان المرحلتين الثانية والثالثة من التوغل العضلى أما فى سرطان المرحلة الأولى فتداخل عددها مع النسيج الطبيعى وفيما يتعلق بإظهار انزيم ميتالوبروتينيز-9 فقد ازدادت نسب اظهاره فى حالات السرطان منه فى النسيج الطبيعى كذلك تزداد معدلات ظهوره مع زيادة درجة ومرحلة توغل السرطان وتكون الزيادة أكثر وضوحاً عند تقدم السرطان من الدرجة الثانية الى الثالثة ومن الأولى الى الثانية وعند توغله من المرحلة الثانية الى الثالثة وبالمقارنة فقد ظهر هذا الإنزيم بنسبة ضئيلة فى حالتين فقط من حالات النسيج الطبيعى. أخيراً أوضحت الدراسة أن هذا الإنزيم تظهرة كل من الخلايا السرطانية وبعض خلايا النسيج اللحمى

ونستنتج من هذه الدراسة حدوث توالد الأوعية الدموية الدقيقة مع تحلل النسيج الواقع بين الخلايا فى نفس الوقت مع تصاعد درجة ومرحلة توغل السرطان، كما نستنتج تعاون الخلايا السرطانية وبعض خلايا النسيج اللحمى الغير سرطانية فى استفحال هذا النوع من الأورام.