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Involvement of Calcineurin in the Pathophysiology of Endometrial Cancer

F. P. Sarukhanyan, G. A. Hovhannisyan, O. V. Hunanyan, N. H. Barkhudaryan

H. Buniatian Institute of Biochemistry NAS RA
0014, Yerevan, 5/1 P. Sevag str.

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Endometrial cancer (EC) is the 3-rd leading type of cancer among women in Armenia [22]. Post-menopausal women are mainly affected, with ~80% of patients being over 55 years of age. The normal cycling endometrium is considered as a site of regular, repeated inflammation and the contribution of inflammation to the initiation and progression of endometrial adenocarcinoma is discussed by Wallace et al. [23]. Today, the causal relationship between inflammation, innate immunity and cancer is more widely accepted [16]. However, many of the molecular and cellular mechanisms mediating this relationship remain unresolved. Furthermore, immune environment in EC has been less characterized than in other tumors such as breast cancers.

Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase 2B or calcineurin (CN) is a key enzyme leading to the activation of the immune system by participating in the synthesis of several cytokines (interleukin (IL)-2, tumor necrosis factor (TNF)-α, etc.) and other regulatory proteins via dephosphorylation and activation of NFAT (nuclear factor of active T cells) transcription factors [7,20]. CN has been reported to demonstrate diverse activities in the pathophysiology of cancer. The reduced activity was observed in cervical cancer [14], while in lymphoblastic leukemia [12], colorectal [15] and ovarian cancers [19] increased activity was detected. We hypothesized that such changes in CN activity in pathophysiology of malignant tumors may be not only organ-specific, but also depend on the stage of the disease and the patient's age. Indeed, more recently, we have shown that CN activity in plasma and tumor tissue of patients with primary ovarian cancer has been changed depending on the stage of the disease [19].

The study of the ways for affecting the immune system for cancer treatment purposes has been in the center of scientists' attention since the XIX century. The discovery of the anti-cancer activity of IL-2 has stimulated development of immunotherapy as an individual method for treating certain types of cancer [5, 8]. Nonetheless, toxicity and dual ambivalent activities of

some cytokines (tumor promoting vs. tumor inhibiting) still remain relevant issues [13]. One of the most potent pro-inflammatory cytokines TNF-α may play a role in cancer growth and metastasis by inducing reactive oxygen species which can cause DNA damage and inhibit DNA repair [18]. TNF-α is involved in the cyclic endometrial shedding and regeneration, and increased TNF-α serum concentrations have been reported in patients with endometrial carcinoma [6].

In recent years, the molecular mechanisms underlying reciprocal interactions between tumor components and immune system are in the focus of intensive studies. In this context the study of the changes in CN activity, as a key activator of the immune system, as well as downstream cytokines, will expand our knowledge concerning the bifunctional nature of CN in pathogenesis of EC. Thus, the aim of present study was to investigate the changes in activity of CN, as well as TNF-α and IL-2 levels in plasma and tumor tissue samples of untreated patients with different stages of primary uterine cancer.

Material and Methods

The blood and tumor tissue samples from postoperative material of untreated patients with the I (n=17), II (n=8) and III (n=8) stages of primary uterine adenocarcinoma were provided by the National Centre of Oncology after V.A. Fanarjyan (NCO MH RA). The plasma of healthy donors (n=6) and histologically checked healthy parts of remote tissue (n=9) were used as a control. The protocol was approved by the Research-related Ethics Committee of the H. Buniatian Institute of Biochemistry NAS RA (IRB0001621, IORG 0009782, Ref. let. #5). Histological study of the postoperative material was conducted by the Laboratory of Clinical Pathomorphology at the NCO MH RA. The most cases of uterine cancer were diagnosed as a moderately differentiated adenocarcinoma, and more rarely, well and poorly differentiated adenocarcinoma. Age of patients was ranged from 35-76, and the average age was 61 years. These patients were divided into two groups: premenopausal and postmenopausal. Among the diagnosed patients, postmenopausal women older than 55 years (82.7%) predominated.

Blood (1.5-2 ml) was collected into sodium citrate (3.2%)-coated vacutainer tubes and centrifugated at 1500 rpm for 10 min. Plasma was separated and stored at -32°C. Tissue samples were homogenized with 2.5 volumes of 50 mM Tris-HCI, pH 7.5 buffer, containing 0.05% Triton-X-100, 0.1 mM EDTA, 1 mM DTT, protease inhibitors, and centrifugated at 20000 ***g*** for 60 min at 4ºC. The supernatant was separated and stored at -32°C as well.

The protein content in samples was determined by Bradford assay [2].

Calcineurin activity was measured by spectrofluorimetric assay using 4-methylumbelliferyl phosphate (4-MUP) as a substrate [1]. We have adapted the assay for our research as described before [19]. One unit of enzyme activity is defined as amount of enzyme that caused the formation of 0.1 nM of 4-

methylumbelliferon (4-MU) at 32ºC for 1 h. The quantity of 4-MU was determined fluorimetrically using a Perkin-Elmer MPF-44A spectrofluorimeter (PerkinElmer Inc., USA).

TNF-α and IL-2 levels in plasma and tumor tissue samples were determined using human TNFα ELISA MAX™ kit (BioLegend Inc., USA) and human IL-2 ELISA MAX™ kit (BioLegend Inc., USA), respectively, according to the manufacturer's recommendations. The optical density was measured in each well at the wavelength of 450 nm using LABLine-022 microplate reader (LABLINE Diagnostics, Austria).

The results were expressed as the means±SEM. Statistical analyses were performed using Origin 6.1. Statistical significance was defined at $P<0.05$ and was determined with one-way ANOVA.

Results and Discussion

Changes in CN activity depending on the stage of disease, age of patients and tissue differentiation

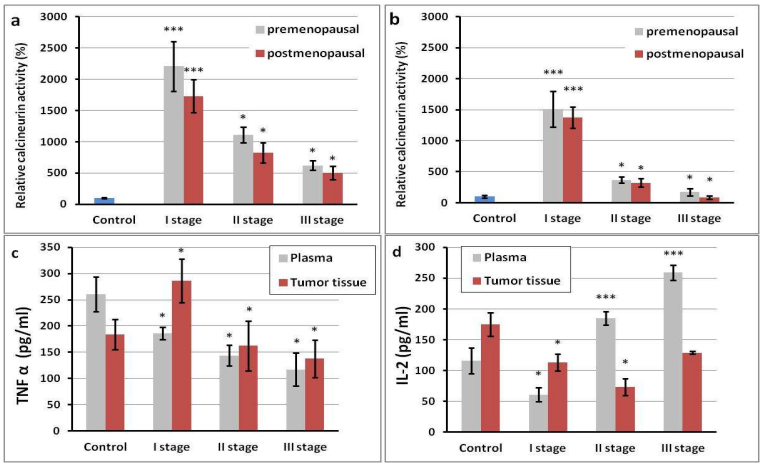

Fig. 1. Calcineurin activity, TNF-α and IL-2 levels in plasma and tumor tissue of the primary uterine cancer patients with the I, II and III stage of disease. Calcineurin activity in plasma (a) and tumor tissue (b) presented for two groups: premenopausal (gray) and postmenopausal (black). *** $P<0,001$ for the I stage compared with control, which considered as 100% and data expressed as % of control; *$P<0,05$ for the II and III stages compared with the I stage. c) TNF-α level in plasma (gray) and tumor tissue (black). *$P<0,05$ for the I stage compared with control, and for the II and III stages compared with the I stage. d) IL-2 level in plasma (gray) and tumor tissue (black). *$P<0,05$ for the I stage compared with control, and for the II stage compared with the I stage. *** $P<0,001$ for the II and III stages compared with the I stage

Data obtained have demonstrated that CN activity in both plasma (Fig. 1a) and tumor tissue (Fig. 1b) of the untreated patients with the primary uterine cancer changed in a parallel manner. CN activity in plasma demonstrated a

sharp increase in the I stage of disease for both premenopausal and postmenopausal groups (22 fold and 17.3 fold, respectively) compared with healthy control group (Fig. 1a). It was shown to be decreased significantly in the II (2 fold for both groups of patients) and III (3.5 fold for both groups of patients) stages of disease compared with the I stage. The same picture was found in tumor tissue samples. CN activity in tumor tissue demonstrated 15 fold and 13.8 fold increase in the I stage of disease for premenopausal and postmenopausal groups, respectively, compared with control group (Fig. 1b). CN activity was shown to be decreased significantly in the II (4 fold for both groups of patients) and III (8.8 fold for the premenopausal group and 16.6 fold for the postmenopausal group, respectively) stages of disease compared with the I stage. Thus, we have found negative correlation (r = -0.6 for plasma and r = -0.72 for tumor tissue, respectively) between CN activity and stage of disease. However, the changes in CN activity in premenopausal and postmenopausal groups were not statistically significant as well as there was no correlation (r = -0.02 for plasma and r = -0.03 for tumor tissue) between CN activity and the age of patients (Fig. 2a, b).

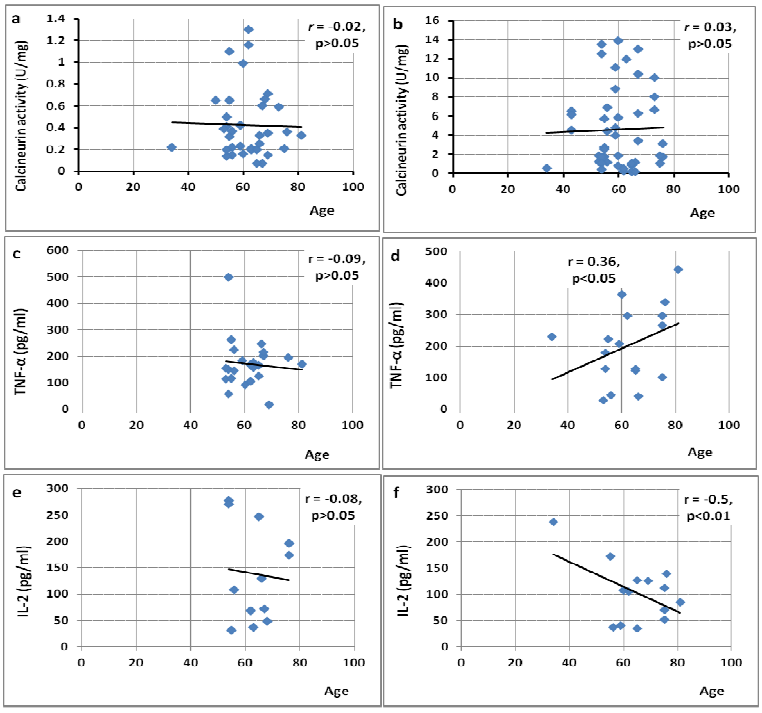

Fig. 2. Scatter plots of the calcneurin activity in plasma (a) and tumor tissue (b), TNF-α levels in plasma (c) and tumor tissue (d), IL-2 levels in plasma (e) and tumor tissue (f) against the age of the primary uterine cancer patients. Calcineurin activity is expressed as unit of activity obtained spectrofluorimetrically per milligram of total protein content (U/mg protein)

Interestingly, we have also found negative correlation between CN activity and tissue differentiation (r = -0.5 for plasma and r = -0.72 for tumor tissue, respectively). As was shown in Fig. 3a, CN activity in both plasma and tumor tissue demonstrated significant increase (16.3 and 22.9 fold, respectively) in case of well differentiated adenocarcinoma compared with the control group. CN activity was shown to be decreased significantly in both plasma (1.8 fold) and tumor tissue (2.3 fold) in case of moderately differentiated adenocarcinoma compared with the well differentiated adenocarcinoma. In case of poorly differentiated adenocarcinoma CN activity demonstrated continued decrease in plasma (2.5 fold) and tumor tissue (8.6 fold) compared with the well differentiated adenocarcinoma as well (Fig. 3a).

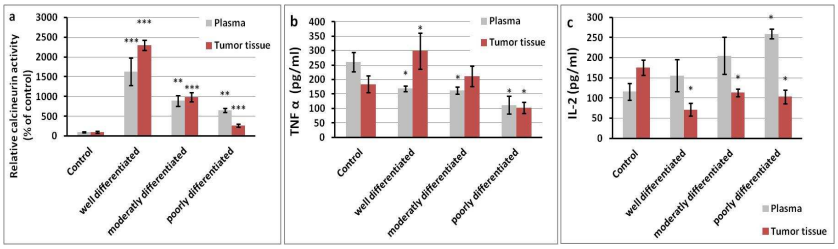

Fig. 3. Calcineurin activity (a), TNF-α (b) and IL-2 (c) levels in plasma (gray) and tumor tissue (black) of the primary uterine cancer patients depending on tissue differentiation. Calcineurin activity expressed as % of control which considered as 100%. *** P<0,001 for the well differentiated adenocarcinoma compared with control, and for the moderately and poorly differentiated adenocarcinomas compared with the well differentiated adenocarcinoma; ** P<0,02 for the moderately and poorly differentiated adenocarcinomas compared with the well differentiated adenocarcinoma (a). *P<0,05 for the well differentiated adenocarcinoma compared with control, and for the moderately and poorly differentiated adenocarcinomas compared with the well differentiated adenocarcinoma (b, c)

Changes in TNF-α level depending on the stage of disease, age of patients and tissue differentiation

TNF-α level in tumor tissue demonstrated 1.55 fold increase in the I stage of disease compared with the control, and continuously decreased in the II (1.76 fold) and III (2.07 fold) stages of disease compared with the I stage (Fig. 1c). In contrary, in plasma TNF-α level demonstrated continuously decrease depending on the stage of disease: 1.4 fold for the I stage of disease compared with the control group, 1.3 fold for the II stage and 1.6 fold for the III stage compared with the I stage, respectively (Fig. 1c). The changes in TNF-α level in premenopausal and postmenopausal groups ware not statistically significant, and there was no correlation (r = -0.09) between plasma TNF-α level and the age of oncologic patients (Fig. 2c). In contrary, there was a positive correlation (r = 0.36, $P<0.05$) between tumor tissue TNF-α level and the age of oncologic patients (Fig. 2d).

We have also detected negative correlation between TNF-α level and tissue differentiation (r = -0.65 for tumor tissue and r = -0.36 for plasma, respectively) (Fig. 3b). TNF-α level in plasma of the patients with EC demonstrated significant 1.55 fold decrease in well differentiated adenocarcinoma compared with the control group. However, there were no statistical significant changes in moderately and poorly differentiated adenocarcinomas compared with the well differentiated adenocarcinoma. In tumor tissue TNF-α level demonstrated significant 1.63 fold increase in case of well differentiated adenocarcinoma compared with the control group. Its levels were shown to be decreased in both moderately (1.4 fold) and poorly differentiated adenocarcinomas (2 fold) compared with the well differentiated adenocarcinoma (Fig. 3b).

Changes in IL-2 level depending on the stage of disease, age of patients and tissue differentiation

IL-2 levels in both plasma and tumor tissue of the patients with uterine cancer have shown modest but statistically significant decrease for the I stage of disease (1.9 and 1.55 fold, respectively) compared with the control group (Fig. 1d). In the II stage of disease IL-2 levels demonstrated significant 3 fold increase in plasma and continued 1.55 fold decrease in tumor tissue compared with the I stage of disease. In the III stage of disease IL-2 levels demonstrated continued increase (4.25 fold) in plasma and modest but not statistically significant increase (1.14 fold) in tumor tissue compared with the I stage (Fig. 1d). The changes in plasma IL-2 level in premenopausal and postmenopausal groups ware not statistically significant. There was no correlation (r = -0.08) between plasma IL-2 level and the age of oncologic patients as well (Fig. 2e). In contrary, there was a negative correlation (r = -0.5) between tumor tissue IL-2 level and the age of oncologic patients (Fig. 2f).

Although, a weak correlation was found between plasma IL-2 level and tissue differentiation (r = 0.29), statistically significant changes (1.66 fold increase) in plasma IL-2 levels were detected only in poor differentiated adenocarcinoma compared with the well differentiated adenocarcinoma (Fig. 3c). In contrary, in tumor tissue IL-2 level demonstrated significant 2.46 fold decrease in case of well differentiated adenocarcinoma compared with the control group. It was shown to be increased in both moderately (1.6 fold) and poorly differentiated adenocarcinomas (1.45 fold) compared with the well differentiated adenocarcinoma (Fig. 3c). However, the changes in IL-2 level in poorly differentiated adenocarcinomas compared with the well differentiated adenocarcinoma were not statistically significant. Despite this, there was a correlation (r = 0.43) between tumor tissue IL-2 level and tissue differentiation.

This is the first study to show an involvement of calcineurin in pathophysiology of EC. The significant increase in CN activity in the I stage of EC indicates on similar increase in the level of proinflammatory cytokines, such as TNF-α, because, CN participates in the synthesis of these cytokines via

activation of NFAT [7, 20]. Indeed, we have found the increase of tumor tissue TNF-α level in the I stage of EC. This could be considered as the very first and rapid response of the host immune system to malignant transformation since the organism uses the inflammation to fight against the neoplasms [21]. However, such a chronic activation of the immune system contributes to chronic inflammation. The immune system, which initially plays a protective role in the onset of tumor, eventually can contribute to the development of the tumor. Indeed, the mechanisms through which the tumor can escape from the immune system are numerous. Many types of tumor cells produce immunosuppressive cytokines and chemokines that negatively regulate the immune system [11]. The existing data are indicating the role of chronic inflammatory milieu in the course of detected immunosuppression during cancer maturation and progression [24]. IL-2 is the key cytokine of anti-cancer immunity regulation [5, 19, 21]. In this context, decrease in IL-2 level, could be explained as an expected immunosuppression driven by inflammation due to the fact that, inflammation, as a double-edged sword, is actively abused by the cancer itself to promote neoangiogenesis and to escape from immune cells attacks [24]. The finding that CN activity is significantly reduced in the third stage of the disease (compared to the first stage), may indicate that by this way tumor cells possibly avoiding from CN driven apoptosis. This point of view is supported by the data that in uterus tumor cells, which demonstrate chemoresistance to doxorubicin, reduced CN expression is observed, which suppresses doxorubicin stimulated apoptosis by preventing the activation of transcription factors NFAT [10]. This is one of the many mechanisms by which the tumor develops self-defense and autonomy. On the other hand, the decrease in CN activity in patients with EC in the third stage of the disease and coming close to control, possibly may be explained by the point of view that changes in CN activity in EC pathology plays an important role only in the early stages of disease, and then the other mechanisms are involved to contribute to the tumor development.

Results obtained in this study demonstrated that the levels of TNF-α generally changed in accordance with the CN activity. This was mainly expected since calcineurin, as already mentioned, is involved in the synthesis of TNF-α [7, 20]. Furthermore, there is a growing amount of evidence concerning the anti-inflammatory and, at the same time, tumor promoting role of M2 subtype of tumor associated macrophages (TAMs) [17]. The crucial point of cancer progression is the M1 to M2 shift or polarization caused by critical levels of pro-inflammatory cytokines and other factors [9, 25]. As the expression and production of TNF-α, in the case of M2 subtype prevalence is much lower than for M1 [9], it's not surprising to find a gradual decrease of TNF-α level in the II and III stages of disease as well. To explain the finding that changes in IL-2 levels in plasma and tumor tissue were not entirely consistent with changes in CN activity, it's worth to mention the multifactorial nature of the regulation of IL-2 gene expression [3]. Thus, the changes in IL-2 level perhaps is not only

drawn by CN, but involves some other factors as well. The fact that changes in plasma cytokine levels do not quite correspond to changes in the levels of these cytokines in the tumor tissue indicates that the tumor, in turn, leads its own struggle against organism in order to survive and progress. However, since in this study we examined only changes in CN activity and levels of downstream cytokines, more detailed and in-depth studies are needed to find out the molecular mechanisms of these changes and their relationship.

Further to stage of disease, other factors have been found to influence changes in CN activity and cytokine levels. We have found that changes in CN activity and in TNF-α, IL-2 levels depending on the tissue differentiation correspond to stage-dependent changes in CN activity and cytokine levels. This was not surprising because the low degree of tumor tissue differentiation is most typical for advanced stages of the disease. Interestingly, we have not detected correlation between the changes in CN activity and downstream cytokine levels and the pre- or postmenopausal status of patients, as well as age of patients. The only exception is the changes in the level of cytokines in the tumor tissue depending on the age of patients which are in accordance with the findings reviewed by Decker et al. [4]. Thus, although this study has generated a lot of questions that need to be solved with the help of additional and detailed researches, however, data obtained for the first time reveal the light on the activity of calcineurin in the pathophysiology of EC.

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Вовлечение кальцинейрина в патофизиологию рака эндометрия

Ф. П. Саруханян, Г. А. Оганнисян, О. В. Унанян, Н. А. Бархударян

Рак эндометрия связан как с постоянным воспалением, так и с нарушением регуляции иммунного ответа. Кальцинейрин тесно взаимодействует с компонентами взаимосвязанных иммунной и про/противовоспалительной систем, участвующих в патогенезе рака. В этой работе мы показали участие кальцинейрина в патофизиологии рака эндометрия. Кроме того, полученные результаты продемонстрировали, что изменения активности кальцинейрина, а также уровней взаимосвязанных IL-2 и TNF-α в плазме и опухолевой ткани нелеченых онкологических больных с различными стадиями первичного рака матки зависят от стадии заболевания

и дифференцировки ткани, но не от возраста пациентов. Полученные данные могут быть использованы для пересмотра и корректировки существующих стратегий лечения рака эндометрия.

Կալցինեյրինի ներգրավվածությունը էնդոմետրիումի քաղցկեղի պաթոֆիզիոլոգիայում

Ֆ. Պ. Սարուխանյան, Գ.Ա. Հովհաննիսյան, Օ. Վ. Հունանյան, Ն. Հ. Բարխուդարյան

Էնդոմետրիումի քաղցկեղը կապված է ինչպես շարունակական բորբոքման, այնպես էլ իմունային պատասխանի խանգարման հետ: Կալցինեյրինը և քաղցկեղի պաթոգենեզում ներգրավված և փոխկապված իմունային և նախա/հակաբորբոքային համակարգերի բաղադրիչները սերտորեն փոխազդում են: Այս աշխատանքում ցույց ենք տվել կալցինեյրինի ներգրավվածությունը էնդոմետրիումի քաղցկեղի պաթոֆիզիոլոգիայում: Ավելին, ստացված տվյալները ցույց են տվել, որ կալցինեյրինի ակտիվությունը, ինչպես նաև փոխառնչվող IL-2-ի և TNF-α-ի մակարդակներն արգանդի առաջնային քաղցկեղի տարբեր փուլերում գտնվող չբուժված հիվանդների պլազմայում և ուռուցքային հյուսվածքում փոփոխվում են փուլից և հյուսվածքների տարբերակումից, բայց ոչ հիվանդների տարիքից կախված: Ստացված արդյունքները կարող են կիրառվել՝ էնդոմետրիումի քաղցկեղի հեռանկարային բուժման առկա ռազմավարությունները վերանայելու և ճշգրտելու համար:

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