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PROSTATE CARCINOMA AND GREEN TEA: PSA-TRIGGERED BASEMENT MEMBRANE DEGRADATION AND MMP-2 ACTIVATION ARE INHIBITED BY (−)EPIGALLOCATECHIN-3-GALLATE

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Prostate-specific antigen (PSA) is a serine-protease that, in addition to cleaving semenogelins in the seminal coagulum, is able to cleave extracellular matrix glycoproteins, thereby affecting cell migration and metastasis. We here report some new activities of PSA that deserve careful consideration in the cancer context: degradation of gelatin, degradation of type IV collagen in reconstituted basement membrane (Matrigel) and activation of progelatinase A (MMP-2), but not pro-MMP-9, in a cell-free system. Since consumption of green tea has been reported to lower the risk of prostate cancer, we investigated the effects of the major flavanol of green tea, (−)epigallocatechin-3-gallate (EGCG), on expression and activity of PSA by prostate carcinoma cells. In addition to restraint of PSA expression, EGCG was found to inhibit in a dose-dependent manner all the above PSA activities, at concentrations lower than the cytotoxic serine-protease inhibitor PMSF and close to levels measured in the serum following ingestion of green tea. The activity of PSA was suppressed also by the elastase released by the inflammatory leukocytes. These results highlight new PSA activities, suggest gelatin zymography as a new convenient assay for PSA, propose EGCG as natural inhibitor of prostate carcinoma aggressiveness, but also stimulate further investigation on the role of prostatic inflammation.
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Key words: *prostate-specific antigen; prostate carcinoma; MMP-2 activation; invasion; (−)epigallocatechin-3-gallate*

Prostate-specific antigen (PSA) is a kallikrein-like serine-protease constitutively secreted by normal epithelium of the prostatic gland as an inactive 244 amino acid precursor protein, which is subsequently cleaved to the active enzyme of 33 kDa.[1] PSA is a major protein in semen where it cleaves the 2 most abundant proteins, semenogelin and fibronectin, thus triggering liquefaction of the seminal plasma coagulum[2] and facilitating spermatozoa progression.

PSA, whose expression is regulated by androgens,[3,4] has been found to be raised in benign prostatic hyperplasia[5] and neoplasia,[6] and its measurement has come into wide use for early detection of prostate cancer.[7] Higher expression levels of other proteases, such as the matrix metalloproteinase-2 (MMP-2), are associated with increasing Gleason score and aggressive behavior of prostate cancer.[8] Thus, the demonstration that PSA may degrade extracellular matrix components such as laminin, along with the restraint of *in vitro* invasion by anti-PSA antibodies,[9] leads to the suggestion that PSA may directly contribute to the invasive ability of prostate cancer cells, also via an MMP-independent pathway.[10] This makes PSA not only a marker for early detection but also a target for prevention and intervention in prostate cancer, and serine-protease inhibitors (*e.g.*, serpins) have been suggested as useful agents for blocking the progression of preinvasive to invasive carcinoma.[10]

Some vegetable secondary metabolites such as catechins, abundant in green tea, have already been reported to inhibit various metallo- and serine-proteinases instrumental to inflammation, angiogenesis and cancer invasion,[11,12] especially MMP-2, MMP-7, MMP-9 and leukocyte elastase (LE).[13–17] A number of epidemiologic observations have led to the association of green tea consumption with prevention of cancer development and spread,[18] and some studies indicate a chemopreventive effect of green tea on prostate carcinoma (PCa).[19] Epidemiologic surveys register the lowest prostate cancer incidence in Japanese and Chinese populations, consumers of green tea,[20,21] and a very recent case-control study in southeastern China reports that the risk of PCa declines with increasing frequency, duration and quantity of beverage consumption, with an odds ratio (relative to nontea-drinkers) down to 0.13 for those drinking tea for more than 40 years, and 0.09 for those consuming more than 1.5 kg of tea leaf yearly.[22] Another major observation is that the main catechin contained in green tea, (−)epigallocatechin-3-gallate (EGCG), has been shown to be responsible for growth inhibition and regression of human prostate tumor in athymic nude mice.[23]

However, the beneficial antineoplastic properties of green tea reported at epidemiologic level face controversial experimental results. A limited antineoplastic activity, as defined by a decline in PSA levels, has in fact been attributed to green tea by a recent short-term phase 2 clinical trial in patients with androgen-independent metastatic prostate carcinoma.[24] Conversely, while in androgen-sensitive prostate carcinoma cells the hormone boosts the expression of both MMP-2[25] and PSA,[26] tea polyphenols downregulate the expression of the androgen receptor in prostate cancer cells,[27] thus restraining the potential proteolytic machinery.

To contribute to a better comprehension of the molecular events modulated by green tea in the prostate, we undertook a detailed study of the proteolytic potential of PSA on gelatin, type IV collagen in reconstituted basement membrane, and the possibility that it may act as progelatinase activator; we also investigated whether EGCG may act as PSA inhibitor.

Abbreviations: DHT, dihydrotestosterone; ECM, extracellular matrix; EGCG, (−)epigallocatechin-3-gallate; LE, leukocyte elastase; MMP, matrix metalloproteinase; NEM, N-ethyl-maleimide; PCa, prostate carcinoma; PMN, polymorphonuclear phagocyte; PMSF, phenylmethanesulfonyl fluoride; PSA, prostate-specific antigen; TIMP, tissue inhibitor of MMP; uPA, urokinase-type plasminogen activator.

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MATERIAL AND METHODS

Enzymatically active PSA from seminal fluid was from Calbiochem (Darmstadt, Germany), LE (catalog no. S563) from Elastin Products (Ownsville, MO), Matrigel from Collaborative Research, Beckton-Dickinson (Bedford, MA), polycarbonate filters from Millipore (Bedford, MA), affinity-purified anti-uPA neutralizing polyclonal antibodies from Monosan (Uden, The Netherlands). All the other reagents were purchased from Sigma (St. Louis, MO). EGCG was purified $\geq$ 95%.

Cells

Human SK-N-BE neuroblastoma, A2058 melanoma, RD rhabdomyosarcoma, DU145 and LNCaP prostate carcinoma cells, plus pseudoxantoma elasticum (PXE) primary fibroblasts, were used. The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany) except the LNCaP grown in RPMI-1640. The medium contained 100 U/ml penicillin, 100 μg/ml streptomycin, and the cells were incubated in 5% CO_2 in air at 37°C. For production of human gelatinase-containing conditioned medium, the cells were incubated overnight in serum-free medium.

For studying the effect of EGCG on PSA expression, LNCaP cells were incubated 24 hr in serum-free medium supplemented with or without dihydrotestosterone (DHT) in the presence of increasing concentrations of catechin, and the medium was clarified by centrifugation at 500*g* and analyzed as described below.

Zymographic analysis

Without heating, samples underwent electrophoresis in 0.1% gelatin-containing polyacrylamide (% specified in figure legends) gels in the presence of sodium dodecyl sulfate under nonreducing conditions. After electrophoresis, the gels were washed twice for 30 min with 2.5% Triton X-100, incubated overnight at 37°C in Tris buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). When PSA activity was analyzed by gelatin zymography, the incubation buffer was substituted with 50 mM Tris-HCl, 1 M NaCl, pH 7.8 (kinetic buffer).

For gelatinolytic activity inhibition assays, the gel slab was cut into slices corresponding to the lanes, put in different tanks containing the stated concentrations of EGCG (freshly solubilized in deionized water), or phenylmethylsulfonyl fluoride (PMSF; presolubilized in methanol) and incubated as above.

The gels were then stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250 and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis. Digestion bands were quantitated using an image analyzer system with GelDoc 2000 and Quantity One software (Bio-Rad, Hercules, CA).

Matrigel degradation by PSA

The degradative activity of PSA on basement membrane components was analyzed using gelified Matrigel. Matrigel solution was freshly prepared in distilled water at concentration of 0.84 mg/ml, and 50 μl were layered on polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size; Millipore) precoated with 0.1% gelatin. Gelification was obtained at room temperature under air flow and in Petri dishes for better solvent evaporation, and the filters were then transferred into 15 mm wells. EGCG and PSA solutions were then added and the volume was adjusted to 300 μl with kinetic buffer; after 48-hr incubation at 37°C in the presence of 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μg/ml amphotericin B, Matrigel was solubilized with 100 μl of electrophoresis sample buffer, lyophilized, reconstituted in distilled water and analyzed by standard SDS-PAGE and Coomassie Blue staining.

Gelatinase activation by PSA

To study the activity of PSA on progelatinases MMP-2 and MMP-9, aliquots of human progelatinase-containing medium were used.[13] PSA was diluted in 50 mM Tris-HCl, 0.1% Na azide, pH 6.0, and added to the media to reach final concentrations of 0–4 μM in the presence of 5 mM N-ethyl-maleimide (NEM) and 1:50 anti-uPA neutralizing antibodies; the pH was checked and confirmed unchanged in all samples, which were then incubated for 5 hr at 37°C. After mixing with electrophoresis sample buffer 4 ×, aliquots of the samples were analyzed by gelatin zymography. The same procedure was used to assay LE.

Inhibition of gelatinase activation

The aliquots of human progelatinase-containing medium described above were premixed 30 min at 4°C with 5 mM NEM and μM concentrations of PMSF or EGCG, the latter freshly prepared in deionized water. PSA was then added to the mixture and the incubation continued as described above. The samples were then examined by gelatin zymography.

PSA quantitation

Free PSA was assayed using the chemiluminescent immunoassay system Immulite 2000 (Diagnostic Products, Los Angeles, CA) according to the manufacturer's instructions. Samples of media conditioned by LNCaP cells incubated 24 hr with or without DHT were aliquoted in a test unit containing a polystyrene bead coated with monoclonal antibody specific for uncomplexed PSA. After 30-min incubation at 37°C, the unit was rinsed, and an alkaline phosphatase-labeled polyclonal goat anti-PSA antibody was introduced. After a second 30-min incubation period, the unbound enzyme conjugate was removed, and the chemiluminescent substrate added; the light-emitting intermediate produced by the latter was then quantitated using a luminometer. The interassay coefficient of variation was $<$ 7.0%.

RESULTS

Gelatin and Matrigel degradation by PSA

Overnight zymographic assay revealed that PSA is able to degrade the gelatin substrate copolymerized with the polyacrylamide, producing a band of digestion slightly slower than a 30 kDa MW marker (Fig. 1*a*). The degradative potential of PSA was considerably more evident when, according to Niemelä *et al*.,[28] the zymogram was developed in buffer containing 1 M NaCl, instead of 0.0 M NaCl; in this case, 1 μg PSA digested most of the gelatin present in the enzymatic band volume (estimated 22 μg).

PSA activity on a reconstituted basement membrane matrix (Matrigel) was also investigated. After 48-hr incubation at 37°C with gelified Matrigel, 0.5 μM PSA produced clear degradation of both chains of laminin (400 and 220 kDa) and of type IV collagen (Fig. 1*b*).

Dose-dependent activation of pro-MMP-2 by PSA

The activating potential of PSA on gelatinases was studied by preincubating culture media conditioned by different cell lines with and increasing amounts of PSA (0–4 μM) in the presence of NEM as cysteine-proteinase inhibitor and anti-uPA IgG, followed by analysis by gelatin zymography. The media conditioned by SK-N-BE and PXE cells revealed a dose-response decrease of MMP-2 zymogen and a parallel increase of 2 forms of higher relative mobility, very likely corresponding to partially and fully activated enzyme (Fig. 2*a* and *c*). These forms were already visible with less than 0.1 μM PSA, but at concentrations $\geq$ 2 μM PSA, the intermediate form progressively faded and disappeared at 4 μM PSA (Fig. 2*c* and *d*). In contrast, no such activation was detected when the same assay was performed using media conditioned by A2058 and DU145 (Fig. 2*b*), LNCaP and RD cells.

Inhibition of PSA lytic activity by EGCG

Degradation of both gelatin and Matrigel component was inhibited by EGCG. When the gelatin zymograms were developed in the presence of increasing concentrations of this catechin, the gelatinolytic band progressively faded in a dose-dependent manner (Fig. 3); the EGCG IC_{50} was close to 10 μM, approximately

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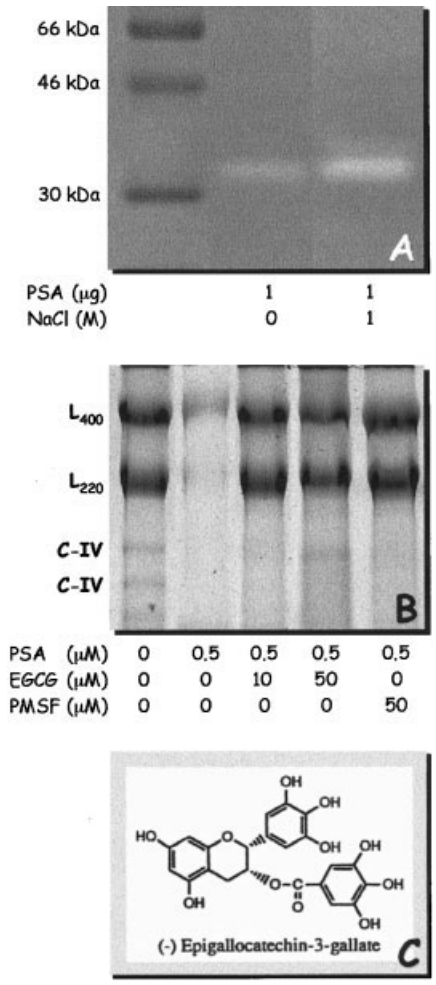

FIGURE 1 – Degradation of gelatin and Matrigel components by PSA. (*a*) PSA gelatinolytic activity as revealed by gelatin zymography (10% polyacrylamide); when the zymogram is developed in buffer containing 1 M NaCl, a more pronounced degradation is achieved. (*b*) the SDS-PAGE (5% polyacrylamide) of Matrigel preincubated 48 hr with PSA shows degradation of both chains of type IV collagen (C-IV) and both chains of laminin (L_{220} and L_{400}). Coincubation with EGCG prevents degradation of type IV collagen more than PMSF. Examples of triplicate experiments. (*c*) Molecular structure of EGCG.

20-fold lower than that of PMSF. Also, Matrigel components were well preserved when 10 and 50 μM EGCG were added together with PSA (Fig. 1*b*, lanes 3 and 4). The inhibition exerted by 50 μM EGCG on the degradation of type IV collagen was slightly higher than that obtained with a same concentration of the serine-protease inhibitor PMSF (Fig. 1*b*).

Inhibition of PSA-triggered activation of pro-MMP-2 by EGCG

The effect of EGCG on the PSA-triggered activation of MMP-2 was studied by preincubating pro-MMP-2-containing medium with 1 μM PSA in the presence of the catechin and NEM, followed by analysis by gelatin zymograpy. A dose-dependent inhibition of MMP-2 activation was registered with an IC_{50} 3 μM and complete inhibition at 10 μM (Fig. 4). Incubation of gelatinase-containing medium with EGCG alone did not alter the control bands.

Restraint of PSA secretion by LNCaP cells in the presence of EGCG

Increase in PSA secretion by androgen-dependent PCa cells and block of its production by flavonoids have already been described,[29] in particular by EGCG in LNCaP 4-day culture.[27] We verified in the present context the effect of EGCG on the 24-hr default and androgen-boosted expression of PSA by LNCaP cells and found that μM concentrations of catechin efficiently restrain PSA secretion in both cases (control cells and cells cultured with 20 nM DHT) without affecting cell viability (not shown). In the conditioned medium without DHT, the level of free PSA fell from 1 ng/ml in the absence of EGCG down to 0.2 ng/ml with 20 μM EGCG; in the medium with 10 or 20 nM DHT, the level of free PSA fell from $>$ 2 to 0.45 ng/ml (Fig. 5).

Differential effect of PSA and LE on progelatinases

Considering the noninfrequent episodes of prostate inflammation and the proteolytic contribution mainly secreted by the infiltrating polymorphonuclear phagocytes (PMNs), a study of the concomitant effect of PSA and LE on tumor progelatinases was carried out using gelatin zymography. When tumor-conditioned medium containing a balanced proportion of pro-MMP-2 and pro-MMP-9 was incubated with 1 μM PSA, MMP-2 was quite efficiently activated, while MMP-9 was almost unaffected (Fig. 6, lane 2). In contrast, incubation with 0.2 mU LE resulted in the partial activation of MMP-9, with no effect on pro-MMP-2 (Fig. 6, lane 5); almost complete suppression of the MMP-2 activity and a more pronounced activation of MMP-9 were registered with 2 mU LE (Fig. 6, lanes 3 and 6). A slightly better preservation of the MMP-9 (but not pro-MMP-2) forms was obtained with the combination of 1 μM PSA and 2 mU LE in comparison to 2 mU LE alone (Fig. 6), but in this case LE completely suppressed the PSA gelatinolytic activity.

DISCUSSION

In addition to its use as early biochemical marker of possible prostate hyperplasia and neoplasia,[5,6] PSA has been suggested as a serious threat in view of its instrumental role in invasive potential of PCa.[8] Restraint of *in vitro* PCa cell invasion is in fact achieved by inhibiting its activity with neutralizing antibodies.[9] This serine-protease has been reported to degrade some extracellular matrix (ECM) components, such as laminin and fibronectin, and activate urokinase-type plasminogen activator (uPA), which may enhance tumor cell invasion.[30] We now add some other ECM macromolecules to the list of PSA potential substrates, propose a new assay for its activity, identify a new and promising natural PSA inhibitor and show that LE suppresses PSA activity.

The first new ECM substrate degraded by PSA is denatured collagen (gelatin), as revealed by developing a 0.1% gelatin zymogram of 1 μg of enzyme. Interestingly, the PSA activity, barely displayed at low ionic strength, is boosted several folds in high ionic strength (1 M NaCl) buffer, as already reported for a synthetic substrate.[28] Expensive chromogenic[31] or fluorogenic[28,32] peptides are used for specific PSA enzymatic assays; but, considering the availability of the natural low-cost substrate, gelatin zymography could be adopted as a convenient alternative assay for monitoring this enzyme, whose activity level can then be quantitated by densitometry. The SDS used for the electrophoresis dissociates most of the enzyme-inhibitor complexes,[33] and its eventual substitution with Triton X-100 in the gel slab, followed by incubation in appropriate buffer, restores the enzymatic activities. In the case of MMP-2 and MMP-9, this assay reveals also the activity of the zymogen forms,[34] but in the case of the active PSA used, possible traces of zymogen would barely be visible, owing to the minimal difference from the active form (only 7 amino acids fewer).[31] The minor gelatinolytic activity with lower relative mobility detectable in some of the presented zymograms should thus be identified; this could correspond to the trypsin-like glandular kallikrein, which typically contaminates the PSA preparations from the seminal fluid,[35] and its potential contribution to the described proteolytic activity should be verified.

The second new substrate degraded by PSA is type IV collagen. Almost complete degradation was obtained by depositing below-

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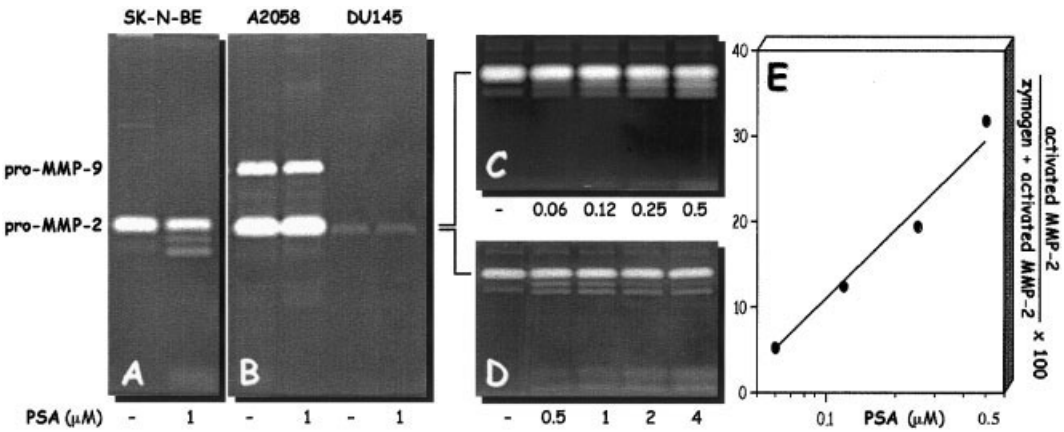

FIGURE 2 – Gelatinase activation by PSA. PSA (1 μM) gives origin to the active (plus and intermediate) form of MMP-2 when incubated with medium conditioned by SK-N-BE cells (*a*), but fails to activate MMP-2 and MMP-9 contained in media conditioned by A2058 or DU145 cells (*b*; 8% polyacrylamide). The MMP-2 in the SK-N-BE medium is activated in a dose-dependent manner by PSA up to 0.5 μM (*c*), but the activation declines from 1 to 4 μM (*d*). (*e*) The graph shows the activation of MMP-2 as quantitated by densitometric analysis of the bands in (*c*). Examples of triplicate experiments.

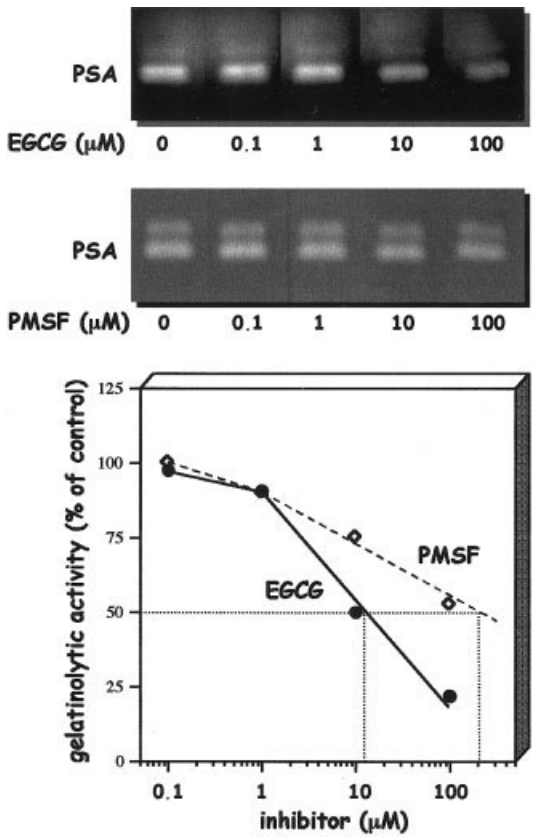

FIGURE 3 – Dose-dependent inhibition of the gelatinolytic activity of PSA by EGCG and PMSF. Fading of gelatinolytic bands of PSA is more evident when the zymography (10% polyacrylamide) is developed in the presence of increasing concentration of EGCG than in the presence of PMSF. The graph of the densitometric values of the digestion bands shows the IC_{50} of EGCG one order of magnitude lower than that of PMSF. Example of duplicate experiment.

micromolar PSA solutions directly onto Matrigel, the reconstituted basement membrane (BM), where type IV collagen and laminin represent the prevalent macromolecules. The degradation of type IV collagen, in addition to that already reported of laminin, reinforces the consideration that this protease may potentially contribute to the invasive aggressiveness of PCa. Gelatin can be generated *in vivo* from different types of collagen in various physiopathologic conditions, but it does not constitute a barrier to cell migration and can be used in chemotaxis assays.[11–13] In contrast, type IV collagen represents the molecular scaffold of the BMs that the cells must actively disorganize in order to cross through during invasion,[36] and in fact type IV collagen-containing reconstituted BM is used in chemoinvasion assays.[11–13] In addition to other proteases such as the metalloproteases MMP-2 and MMP-9,[37] the serine-proteases uPA,[38] leukocyte elastase[16] and proteinase-3,[17] PSA can directly contribute to BM degradation and play an instrumental role in PCa invasive and metastatic aggressiveness.

But PSA can contribute also indirectly, by triggering activation of pro-MMP-2 (but not MMP-9), one of the degradative enzymes chiefly studied for its involvement in tumor invasion.[37] This activation was clearly achieved by incubating PSA with MMP-2-containing media (Figs. 3, 4 and 6), and the potential contribution of uPA[39] or cysteine-proteases to this activation was excluded by preincubation with anti-uPA neutralizing antibodies and NEM, respectively. PSA below 2 μM triggered a remarkable activation in SK-N-BE (neuroblastoma) and PXE (pseudoxantoma elasticum), but was not effective in DU145 and LNCaP (PCa), RD (rhabdomyosarcoma) and A2058 (melanoma) media. We may hypothesize that, in addition to pro-MMP-2, the second set of cells secretes also PSA inhibitor(s) not yet identified, but also that other molecules (TIMPs?) bound to pro-MMP-2 prevent the latter from being attached by PSA. Further investigation is in progress to find the reason for this differential susceptibility, which promises to be of great interest for PCa prognosis, but also to understand why, at concentrations > 2 μM, PSA is apparently no longer effective.

The activity of PSA on both gelatin and pro-MMP-2 was inhibited in a dose-dependent manner by the major flavonoid contained in green tea, EGCG, with IC_{50} close to levels measured in the plasma of moderate green tea drinkers.[40] In comparison to PMSF, one of the routinely used serine-protease inhibitors (not suitable for human use), EGCG is approximately 20-fold more potent in blocking the PSA-triggered degradation of gelatin. The restraint of PSA-triggered activation of pro-MMP-2, together with the direct inhibition of MMP-2 and other proteases instrumental to cell invasion,[11–17] thus contributes a possible mechanistic molecular explanation of the PCa risk decline in high green tea consumers very recently registered in a case-control study.[22]

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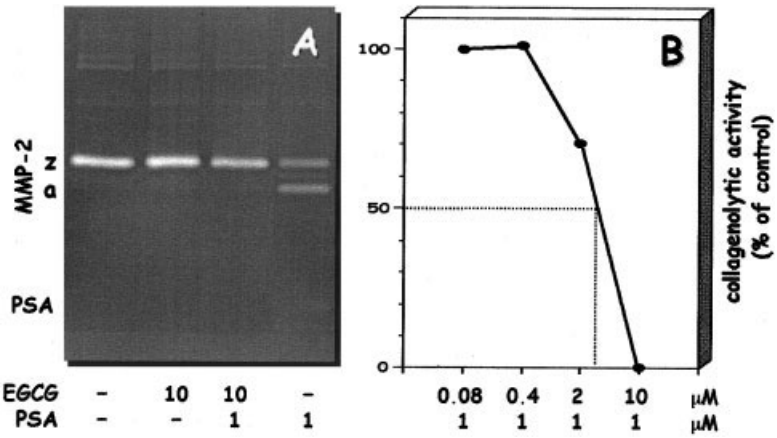

FIGURE 4 – (*a*) MMP-2 activation by PSA is inhibited by EGCG. z, zymogen; a, activated form. (*b*) The graph shows the calculated IC_{50} of a dose-response experiment (8% polyacrylamide); the values are from densitometric analysis of the digestion bands. Example of triplicate experiment.

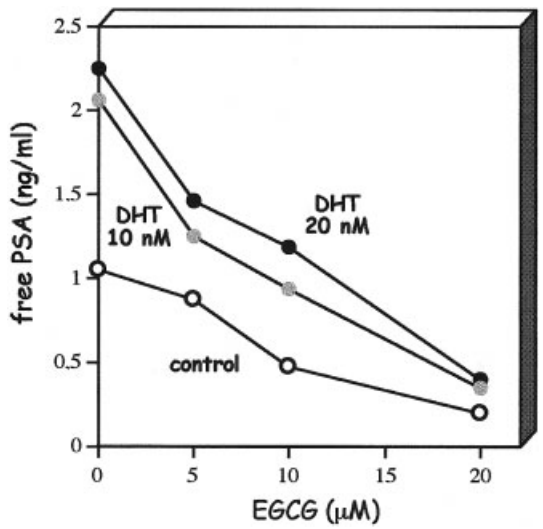

FIGURE 5 – Inhibition of PSA expression by LNCaP cells incubated 24 hr at increasing concentrations of EGCG with or without DHT. Each point represents the average of quadruplicates, with SD $< 7\%$. Example of triplicate experiment.

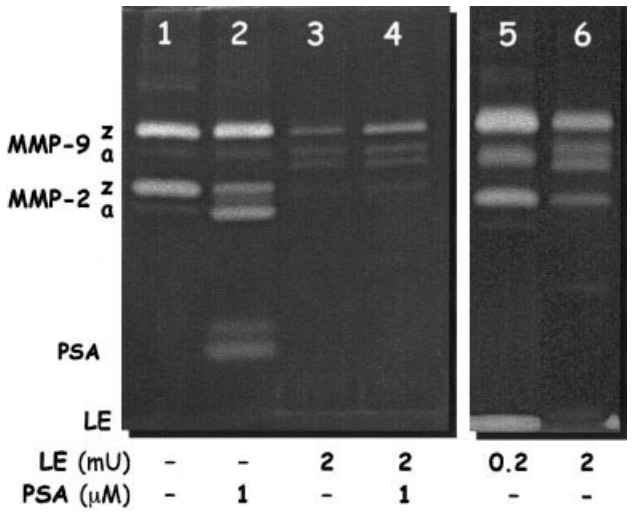

FIGURE 6 – Activation of MMP-2 and MMP-9 gelatinases by PSA in comparison to LE. The zymogen form (z) of MMP-2 is decreased and the active form (a) is augmented, while the zymogen form of MMP-9 remains unmodified following 5-hr incubation with 1 μM PSA. On the other hand, activation of MMP-9 and suppression of the MMP-2 activity are triggered by LE, which eliminates also PSA gelatinolytic activity. Example of duplicate experiment.

In the case of PCa infiltration by inflammatory cells, pro-MMP-2 can be activated also by other proteases released by macrophages and PMNs.[17,41] Generation of the activated form of MMP-2 *in vitro* by proteinase-3, a PMN broad-spectrum serine-proteinase, has recently been reported,[17] though this activity too is inhibited by μM concentrations of EGCG (data not shown), thus preventing pro-MMP-2 activation. Also, LE plays a pivotal role in ECM degradation during inflammation, but fails to activate pro-MMP-2 and, at higher concentrations, has the potential to suppress MMP-2 activity (Fig. 6).[15,41] In contrast, also at high concentrations, LE triggers pro-MMP-9 activation, but the contribution of this metalloprotease to PCa invasion is still to be clarified.

In addition to MMP-2, PSA activity is also radically suppressed in the presence of LE; this suggests that prostate inflammatory involvement might lower the invasive potential of transformed cells. However, leukocyte recruitment, with its load of reactive oxygen species and proteases, very likely represents a more crucial contribution to cell transformation and invasion-favoring matrix modification.[42–44] This recruitment is nevertheless restrained by green tea regimen.[11] With EGCG being the natural, most potent exogenous inhibitor of LE so far identified ($IC_{50} = 0.3$ μM),[16] the LE-triggered suppression of PSA should fall as a consequence.

On the other hand, EGCG exerts on PCa cells a dose-dependent inhibition of the PSA expression, also when this is boosted by DHT.[27] In this case, too, owing to the androgen-dependent expression of PSA and to the triggered repression of the androgen-receptor gene transcription,[27] EGCG is remarkably effective, lowering by approximately 80% the level of secreted PSA[27] without affecting cell viability. Green tea and EGCG may thus exert their beneficial effects on the prostate also by containing the expression of this potentially invasion-triggering protease.

Taking advantage of these biochemical and biologic clues, further investigations are in progress to elucidate the contribution of inflammatory cells to the invasive behavior of PCa cells in the presence of EGCG.

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