Antitumor Effects of Systemic DNAse I and Proteases in an *In Vivo* Model

Catalina Trejo-Becerril, PhD[1], Enrique Pérez-Cardenas, MSc[1],
Blanca Gutiérrez-Díaz, BSc[1], Desiree De La Cruz-Sigüenza, MSc[1],
Lucía Taja-Chayeb, PhD[1], Mauricio González-Ballesteros, BSc[1],
Patricia García-López, PhD[1], José Chanona MD[1],
and Alfonso Dueñas-González, PhD[1,2]

Abstract

Background. Cell-free DNA circulates in cancer patients and induces *in vivo* cell transformation and cancer progression in susceptible cells. Based on this, we hypothesized that depletion of circulating DNA with DNAse I and a protease mix could have antitumor effects. *Study design.* The study aimed to demonstrate that DNAse I and a protease mix can degrade *in vitro* DNA and proteins from the serum of healthy individuals and cancer patients, and *in vivo* in serum of Wistar rats,. Moreover, the antitumor effect of the systemically administered enzyme mix treatmentwas evaluated in nude mice subcutaneously grafted with the human colon cancer cell line SW480. *Results.* The serum DNA of cancer patients or healthy individuals was almost completely degraded *in vitro* by the enzymatic treatment, but no degradation was found with the enzymes given separately. The intravenous administration of the enzymes led to significant decreases in DNA and proteins from rat serum. No antitumor effect was observed in immunodeficient mice treated with the enzymes given separately. In contrast, the animals that received both enzymes exhibited a marked growth inhibition of tumors, 40% of them having pathological complete response. *Conclusion.* This study demonstrated that systemic treatment with DNAse I and a protease mix in rats decreases DNA and proteins from serum and that this treatment has antitumor effects. Our results support the hypothesis that circulating DNA could have a role in tumor progression, which can be offset by depleting it. Further studies are needed to prove this concept.

Introduction

The current model for developing new cancer drugs is based on targeting specific molecular alterations in tumors. In general, this approach has yielded modest improvements in survival[1-3]; therefore, it is clear that although efforts must continue in this direction, new paradigms of cancer treatment are needed. So far, most research in malignant tumors is directed toward understanding how cancer cells grow and metastasize, though most recently, the tumor microenvironment, which comprises immune cells, vascular cells, lymphatic endothelial cells, and cancer-associated fibroblastic cells, is also the focus of intense research.[4,5] Beyond tumor cells and the microenvironment, we suggest the presence of a so-called malignant circulome, which may serve as a source of systemic circulating molecules that could modulate tumor growth. However, it is yet uncharacterized because of the

absence of experimental models accounting for the systemic nature and complexity of the phenomenon.

The current paradigm in cancer progression is that it occurs via vertical gene transfer; this means that the offspring of an initiating tumor cell inherit the genetic and epigenetic alterations leading to tumor progression. This model, however, ignores that horizontal or lateral transfer

[1]Instituto Nacional de Cancerología, México City, Mexico
[2]Instituto de InvestigacionesBiomédicas, Universidad Nacional Autónoma de México UNAM/Instituto Nacional de Cancerología, México City, Mexico

Corresponding Author:
Alfonso Dueñas-González, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México UNAM/Primer Piso, Edificio de Investigación del Instituto Nacional de Cancerología, San Fernando 22, Tlalpan. CP 14080, México City, Mexico.
Email: alfonso_duenasg@yahoo.com

of DNA, which is carried out by almost all cell types, including tumor cells, may potentially act as an endocrine or paracrine messenger, able to affect the functionality of recipient cells.[6]Increasing evidence suggests a key role for these messengers, which have been characterized as exosomes, microvesicles, apoptotic bodies, and virtosomes.[7-9] Regardless of how extracellular DNA is present in circulation, it has been proposed that circulating DNA could participate in the development of metastases via passive transfection-like uptake of such nucleic acids by susceptible cells.[10] In 1994, Anker et al[11] first demonstrated that the supernatant of cultured human colon cancer cell line SW480 was able to transform recipient immortal murine NIH3T3 cells, which acquire human mutated *K-ras.* Transformation of these recipient cells by plasma of colon cancer patients has been reported as well.[12] Our research group has confirmed that the supernatant of malignant cells and serum of patients with cancer transform immortalized murine cells and that this process is associated with transfer of DNA. Interestingly, we demonstrated that the depletion of DNA in either supernatant or serum requires the concomitant use of DNAse I and proteases because the circulating DNA is protected from DNAse for its association with lipoproteins in virtosomes[9] and that its depletion offset its transforming ability *in vitro*. In addition, we have demonstrated tumor progression in immunocompetent rats xenografted with human colon cancer cells as a source of circulating DNA, which were pretreated with the carcinogen dimethylhydrazine. Furthermore, there was a suggestion that systemic treatment with DNAse I and proteases prevents tumor progression in these rats.[13] Here, we demonstrate that the systemic treatment with DNAse I and proteases has an antitumor effect in a model of nude mice grafted with a human malignant cell line.

Material and Methods

Serum Collection and Preparation From Healthy Women and Breast Cancer Patients

Sera were extracted from the blood of 4 women with breast cancer aged 40, 43, 45, and 49 years and 4 healthy women aged 38, 42, 47, and 38 years. Blood was obtained from a peripheral vein in 2 vacutainer tubes (Becton Dickinson, 368162) containing clot-activation additive and a barrier gel to isolate serum. The blood was kept at 4°C, processed within 2 hours, and centrifuged at 400*g* for 20 minutes (Biofuge primo R, Heraeus) at room temperature; serum was collected and passed through a 0.45-µm filter (Sartorius, 16555) to remove cells. The samples were stored at −80°C for subsequent assays. Blood samples were obtained with written consent from source patients and healthy donors.

In Vivo Experiments in Rats

Male Wistar rats weighing 250 to 300 g (HSD: Wistar, Harlan Laboratories) were divided into 2 groups of 4 animals each. Group 1 was inoculated (in each flank) subcutaneously with 2×10^6 C6 cells (C6 rat glioma cell line from ATCC) resuspended in 100 µL of serum-free culture medium (DMEM-F12). Group 2 corresponded to the control group, which was not inoculated with cells. Sizes of tumors were measured with an electronic caliper, and tumor volume was calculated using the following formula: V (mm^3) $= a \times b^2 \times (\pi/6)$, where V is the volume, a the major diameter, and b the minor diameter. Extraction of serum of rats was performed by bloodletting from the tail caudal vein cannulation. Immediately, 500 µL of whole blood was taken (in tubes without anticoagulants [Terumo]), and the enzyme treatment was subsequently administered as described below. After treatment, 500 µL of whole blood were taken at increasing times: 7.5 minutes, 15 minutes, 30 minutes, 60 minutes,and 6 hours. For blood samples taken at 24, 48, 72, and 96 hours, we used the retro-orbital under light anesthesia. All blood samples were incubated at 4°C for 2 hours and then centrifuged at 4°C at 1000*g* for 20 minutes. Serum was collected and filtered with a 0.45-µm filter. The samples were stored at −80°C for subsequent assays.

Enzymatic Treatment of Serum In Vitro

We first determined the concentration of total protein in serum using the bicinchoninic acid assay,[14] and then, serum was digested with the protease mix. Briefly, 100 µL of serum was incubated first with a mixture of papain (31.25 µg/100 µL; Sigma) + chymotrypsin (12.5 µg/100 µL; Sigma) + trypsin (12.5 µg/100 µL; Sigma) at 37°C for 1 hour, then inactivated at 56°C for 30 minutes. Then, the sample was incubated with DNAse I (Sigma) at a concentration of 143.75 µg/100 µL for 1 hour and immediately inactivated at 65°C for 30 minutes. The integrity of the proteins present in the serum at the end of the protein degradation assay was determined by SDS-polyacrylamide gel electrophoresis at 7.5% or 12.5% concentration; then, gels were stained with Coomassie blue and photographed.

For the analysis of serum DNA, its concentration was determined using the modified technique of Goldshtein et al[15] to measure DNA directly in serum with SYBR Gold. Briefly, samples were diluted to 40% with PBS and were measured in a fluorometer (excitation wavelength of 488 nm and emission wavelength of 535 nm). In addition, serum DNA was extracted by SDS/proteinase K digestion followed by phenol/chloroform extraction as described by Anker et al.[11] Briefly, 500 µLof serum was mixed with 500 µLof a solution of SDS/proteinase K (Invitrogen) and incubated overnight at 55°C. An equal volume of phenol/chloroform (1:1 v/v) was added, vortexed briefly, and centrifuged

at 800*g* for 10 minutes (Biofuge primo R, Heraeus). The aqueous phase was recovered and mixed with an equal volume of chloroform and centrifuged at 800*g*(Biofuge primo R, Heraeus) for 5 minutes. The aqueous phase was precipitated overnight at −20°C with 1/10 volume of 7.5M ammonium acetate, 1 μLof glycogen, and 2.5 volumes of 100% ethanol and then centrifuged at 1200*g*(Biofuge primo R, Heraeus) for 45 minutes. The DNA pellet was washed with 70% ethanol, air dried, and resuspended in nuclease-free water. Extracted undigested and digested serum DNA was electrophoresed in 1.5% agarose gel stained with GelRed (Biotium Inc) for visualization.

Treatment In Vivo With DNAse I and Proteases

Rats with and without tumor were treated with the enzyme mix at doses previously reported[13]: papain (25 mg/kg) + trypsin (10 mg/kg) + chymotrypsin (10 mg/kg) + DNAseI (2.3 mg/kg). The mean tumor volumes of tumor-bearing rats were 1421 ± 812 mm³ (left tumor) and 1665.06 ± 673.34 mm³ (right tumor). Enzymes were administered once through a stent in the tail vein, and blood samples from all animals were obtained before treatment and at 7.5, 15, 30, and 60 minutes after treatment.

Antitumor Effect of Enzymes in Nude Mice

Athymic *BALB/c* mice (*nu/nu*) females (Harlan Laboratories) of 6 weeks of age were divided into 4 groups. Each group consisted of 6 animals injected subcutaneously with 1×10^6 SW480 cells (human colon cancer cell line, American Type Culture Collection) suspended in 100 μLof serum-free culture medium. The groups were as follows: (1) SW480 cells injection as positive control; (2) SW480 cell injection plus treatment with a protease mix (trypsin, chymotrypsin, and papain: 5, 5, and 12.5 mg/kg,respectively)[16]; (3) SW480 cell injection plus treatment with DNAse I (2.3 mg/kg)[17]; and (4) SW480 cell injection plus treatment with proteases mix plus DNAse I.[13] Treatment with DNAse I was intramuscular, whereas the mixture of proteases was administered via the intraperitoneal route. All enzymes were diluted with saline solution. The enzymes were administered daily for 8 weeks starting at day 21 after tumor implantation. Clinical signs, weight, and tumor size were registered weekly. The size of the tumors was measured with an electronic caliper and size-volume was estimated using the following formula: V (mm³) $= a \times b^2 \times (\pi/6)$, where V is the volume, a the major diameter, and b the minor diameter. At the end of the treatment period, mice were killed humanely, and tumors were removed; in the absence of tumor, the entire site of implantation was sectioned (at least 3 tissue sections) and processed for routine histopathological analysis with hematoxylin and eosin stain. Pathological complete response was defined as the absence of viable tumor cells. An identical experiment using the same number and groups of animals was repeated to confirm the findings. The tumor growth curve includes the 12 animals by group because the results of the 2 experiments were quite similar. Ethical approvals were obtained from the Institutional Research Ethics Board for blood human samples and the Animal Care Committee.

Results

In Vitro Degradation of DNA and Proteins in Serum From Healthy Individuals and Cancer Patients

Previous studies have shown that supernatant DNA can be degraded by *in vitro* treatment with a combination of DNAse I and proteases. Figure 1A confirms that serum DNA of healthy individuals is almost completely degraded by combining DNAse I and proteases ($P<0.001$), whereas DNAse I alone induces no or minimal degradation. Interestingly, the proteases mix alone also induced degradation, which was inferior to that achieved by both types of enzyme but still statistically significant ($P<0.001$). A very similar picture was observed when the serum of breast cancer patients was digested as above, indicating that DNA degradation occurs to the same degree, whether the serum comes from healthy individuals or cancer patients. It can be observed that the basal concentration of DNA in cancer patients was higher as compared with healthy individuals ($P<0.005$; Figure 1B). To corroborate these findings, DNA was extracted from digested and undigested serum and run in agarose gels. As can be seen in Figure1C, lane 1 shows that most DNA was degraded, as compared with undigested serum in lane 2.

The protease mix also degrades serum protein as could be expected, and Figure 2A demonstrates that serum proteins from healthy individuals showed a significant decrease ($P<0.01$), as measured by the bicinchoninic method (similar results observed in the serum of cancer patients, Figure 2B). The decrease was corroborated in acrylamide gels, which showed the pattern of degradation in healthy individuals and in cancer patients (Figures 2C and 2D). Interestingly, the effect is most noticeable in the electrophoretic analysis, which shows almost complete degradation, whereas the measurement by the bicinchoninic method in both healthy individuals and cancer patients shows only about half the degree of reduction.

In Vivo Degradation of DNA and Proteins in Serum of Rats With and Without Tumors

As we have hypothesized that the circulating DNA *in vivo* is responsible for tumor progression in the rat model,[13] we wanted to determine whether the systemic treatment of rats with both DNAse I and the protease mix (papain, chymotrypsin, and trypsin) administered by the intravenous route is active
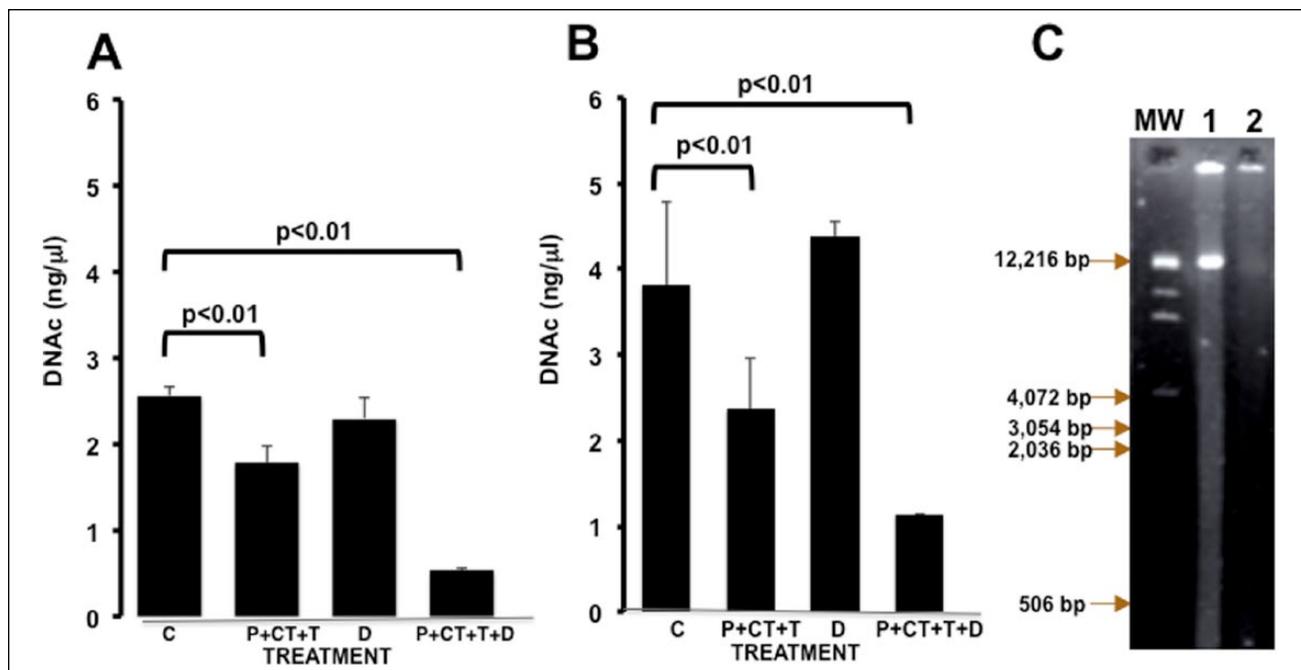
Figure 1. A. DNA concentration in serum of healthy subjects (DNAc), treated *in vitro* with the enzyme mix. Control serum without enzymatic treatment (C); C + papain (31.25 mg/100 mL; P) + chymotrypsin (12.5 mg/100 mL; CT) + trypsin (12.5 mg/100 mL; T); C + DNAse I (143.75 mg/100 mL; D); Dunnett's test. B. As in Figure 1A but in the serum of the breast cancer patients (Dunnett's test). C. Agarose gel electrophoresis of purified DNAc from the serum of a breast cancer patient. The serum from a breast cancer patient was treated *in vitro* with the enzyme mix and then purified and gel electrophoresed.
Abbreviations: MW, molecular weight marker; 1, serum of patient with breast cancer; 2, serum of patient with breast cancer, treated with papain (31.25 mg/100 mL) + chymotrypsin (12.5 mg/100 mL) + trypsin (12.5 mg/100 mL) + DNAse I (143.75 mg/100 mL).

in reducing circulating DNA and proteins from serum. As shown in Figure 3A, the treatment led to a mild but statistically significant reduction starting at 7.5 minutes, which further decreased almost to half the concentration at 15 minutes, and then reaching almost basal levels at 30 and 60 minutes. As observed in the *in vitro* digestion of serum (Figures 1A and 1B), *in vivo* treatment also statistically significantly reduced serum DNA in the rats bearing C6 glioma tumors. In this case, the maximum decrease was observed at 7.5 minutes. It should also be noted that basal serum DNA was higher in the tumor-bearing rats as compared with normal rats ($P>0.2$; Figure 3B).

The effect of the enzymatic treatment on serum proteins was also evaluated in rats. As shown in Figure 4A, when protein concentration was measured by bicinchoninic acid there was a decrease starting at 7.5 minutes, reaching an almost one-third reduction at 15 minutes, and then restoring to close-to-basal levels; all these differences were statistically significant. These changes were well correlated when proteins were analyzed by gel electrophoresis (Figure 4B) at these time points. Similar effects on proteins were shown in the rats bearing the C6 xenograft (not shown). These data clearly demonstrated that the enzyme combination of DNAse I and the protease mix administered systemically decrease circulating serum DNA and serum proteins.

Antitumor Effect of the Enzymatic Treatment in Nude Mice

To demonstrate whether the enzyme combination could have antitumor effects, *nu/nu* mice injected with human colon cancer cells were treated with the enzyme combination systemically. All mice developed measurable tumors. Figure 5 shows that whereas no difference in tumor growth was observed with DNAse I alone or the protease mix alone as compared with controls, the animals that received both DNAse I and proteases exhibited a marked growth inhibition of tumors starting at day 21 of treatment ($P = 0.011$). In fact, 40% (5 out of 12 mice) had a pathological complete response as evaluated with 3 tissue sections. Under these conditions of evaluation, no viable tumor cells and only minimal fibrosis were found. All tumors showed epithelioid histology, and in those treated with the protease mix and DNAse I that did not have complete pathological response, the percentage of necrosis varied between 80% and 90%, and there were scattered viable tumor cells surrounded by stromal atypia and lymphoid infiltrate. Tumors from the protease mix alone exhibited a necrosis percentage between 10% and 30%, whereas in those treated with DNAse I alone, the percentages varied between 5% and 20%, and no changes in stroma were observed.
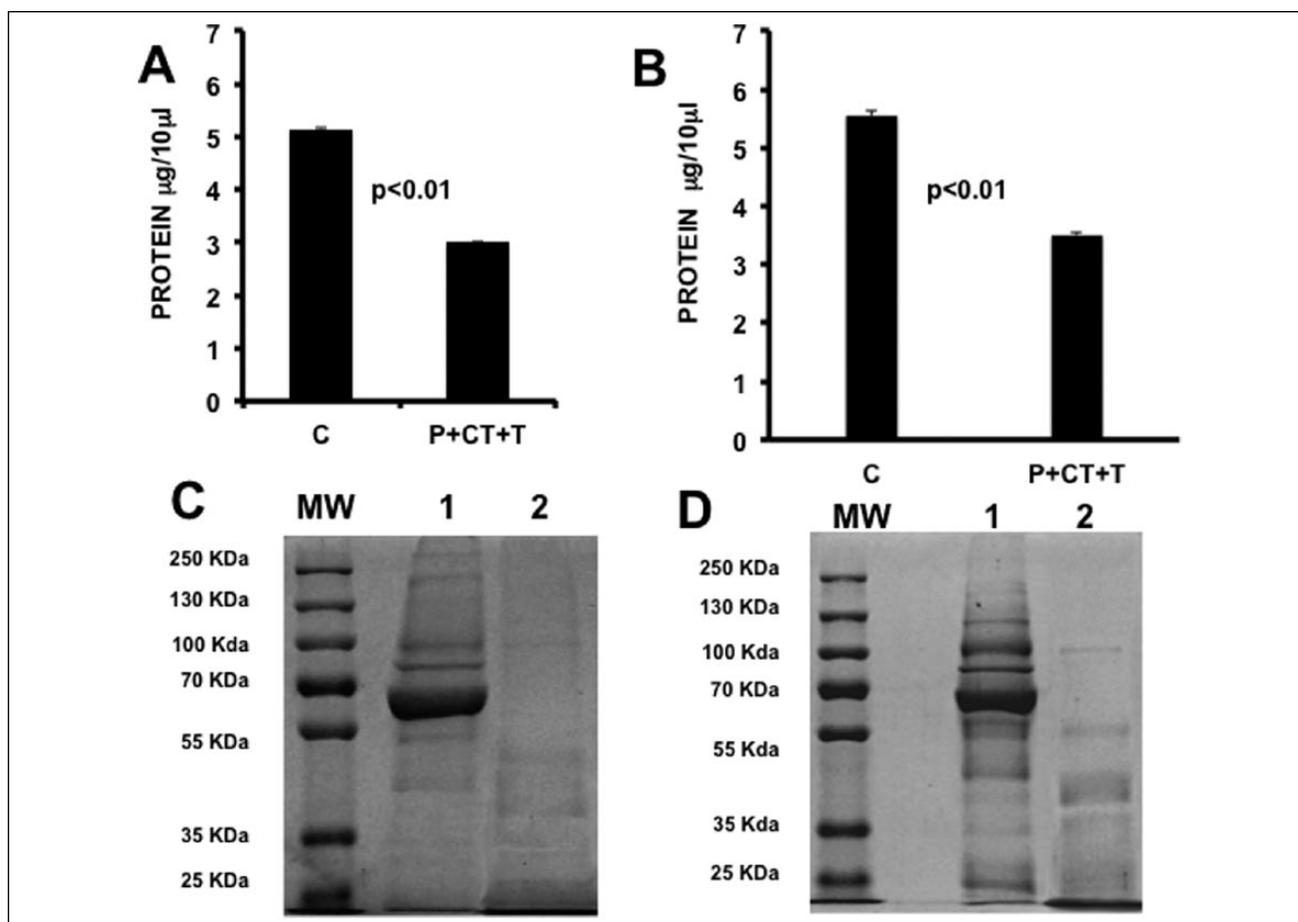
Figure 2. A. Protein concentration in serum of subjects treated in vitrowith the enzyme mix. C: serum from healthy subjects; P+CT+C: control serum treated with papain (31.25 mg/100 mL; P) + chymotrypsin (12.5 mg/100 mL; CT) + trypsin (12.5 mg/100 mL; T); Dunnett's test.B. As in Figure 1A but in the serum of breast cancer patients (Dunnett's test). C. Polyacrylamide gel electrophoresis of the serum samples treated *in vitro*with the enzyme mix; serum from healthy subjects. D. Polyacrylamide gel electrophoresis of the serum samples treated *in vitro*with the enzyme mix; serum from breast cancer patient.
Abbreviations: MW, molecular weight marker; 1, untreated serum; 2, serum treated with papain (31.25 mg/100 mL) + chymotrypsin (12.5 mg/100 mL) + trypsin (12.5 mg/100 mL).

No differences in weight were observed among the groups. Basal and final weights from untreated tumor-bearing mice were 27.73 ± 2.20 g and 28.2 ± 2.97 g, whereas these were 26.82 ± 2.99 g and 26.36 ± 2.29 g in mice treated with the enzyme combination. It is important to note that this experiment was done twice under identical conditions, and because the results were very similar, the tumor growth curve is shown in a single figure.

Discussion

The results of this study show that the systemic administration of DNAse I and a protease mix containing trypsin, chemotrypsin, and papain decreases the levels of DNA and proteins in rats and that the enzymes have antitumor effects in nude mice xenografted with a human colon cancer cell line.

The rationale for use of the enzyme combination (protease mix and DNAse I) was supported by our results *in vitro* and *in vivo*. As shown in Figures 1A, 1B, and 1C, the results clearly suggest the more effective degradation of serum DNA with the combination as compared with either proteases or DNAse I alone, supporting the view that circulating DNA is protected from DNAse for its association with lipoproteins in virtosomes.[9] The results in serum DNA depletion in both healthy and tumor-bearing rats, as shown in Figure 3, indicate that the enzyme combination is active *in vivo*, though it is important to undertake pharmacokinetic and pharmacodynamic studies of both DNAse I and proteases *in vivo* to better understand the kinetics of reduction in DNA in normal and tumor-bearing hosts. This would help explain why there is an apparent difference in the kinetics of DNA reduction (maximum decrease at 15 minutes in healthy and 7.5 minutes in tumor-bearing rats).
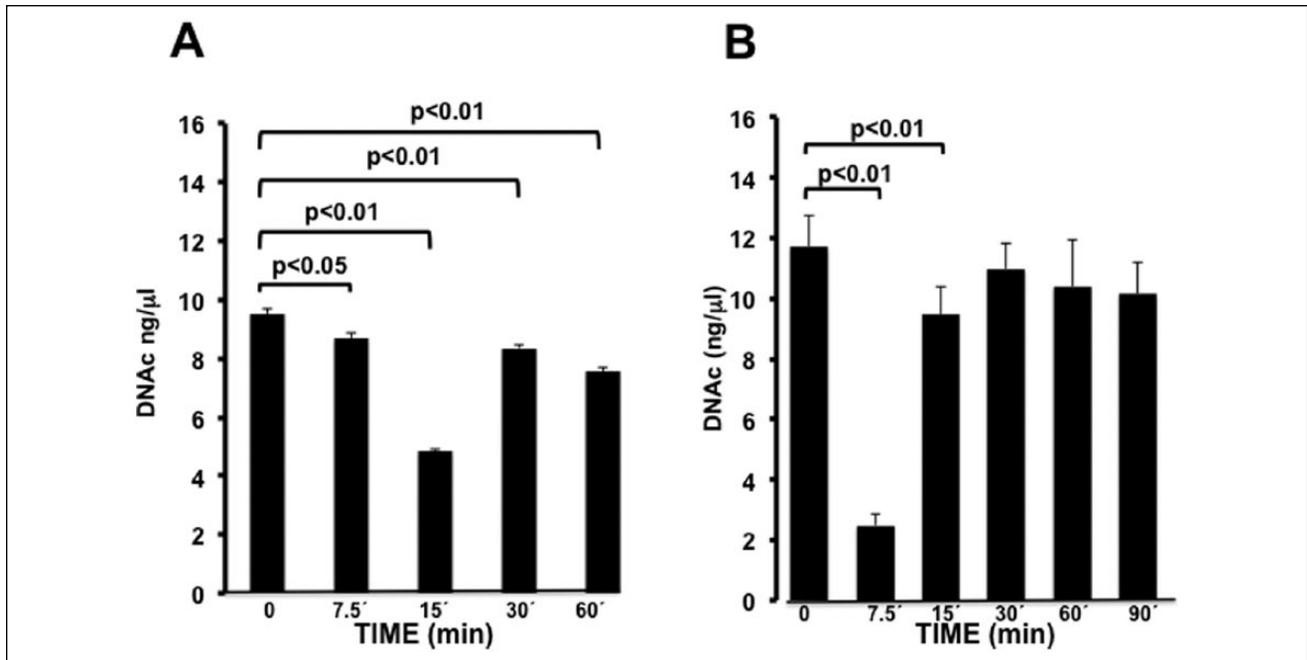
Figure 3. A. DNA concentration in serum of Wistar rats treated with the enzyme mix (papain: 25 mg/kg + trypsin: 10/kg + chymotrypsin: 10 mg/kg + DNAse I: 2.3 mg/kg). Blood samples were taken at 4 different time intervals after treatment. B. DNA concentration in serum of Wistar rats with tumor treated with the enzyme mix (papain: 25 mg/kg + trypsin: 10 mg/kg + chymotrypsin: 10 mg/kg + DNAse I: 2.3 mg/kg). Blood samples were taken at 5 different time intervals after treatment.
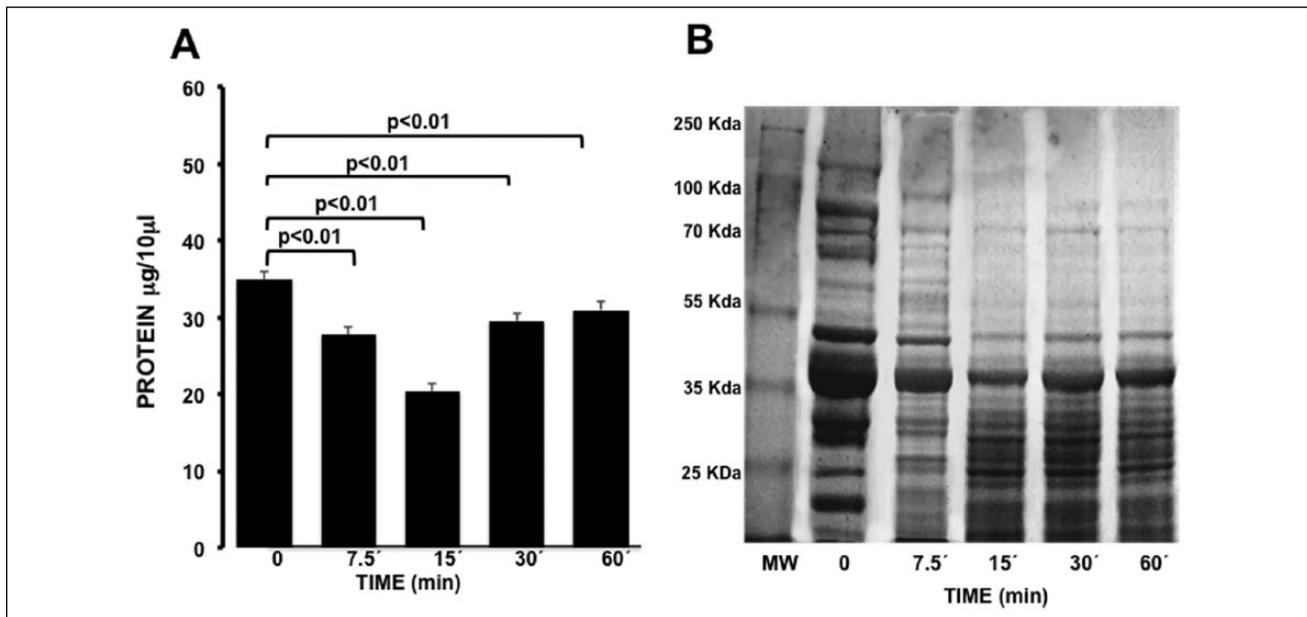Abbreviation: 0, untreated control serum (Dunnett's test).

Figure 4. A. Protein concentration in serum of Wistar rats treated with the enzyme mix (papain: 25 mg/kg + trypsin: 10 mg/kg + chymotrypsin: 10 mg/kg + DNAse I: 2.3 mg/kg). Blood samples were taken at 4 different time intervals after applying the treatment. B. Polyacrylamide gel at 12.5% from Wistar rats that were treated with the enzyme mix (papain: 25 mg/kg + trypsin: 10 mg/kg + chymotrypsin: 10 mg/kg + DNAse I: 2.3 mg/kg) showing serum protein degradation. Blood samples were taken at 4 different time intervals after applying the treatment.
Abbreviation: 0, untreated control serum (Dunnett's test); MW, molecular weight marker.
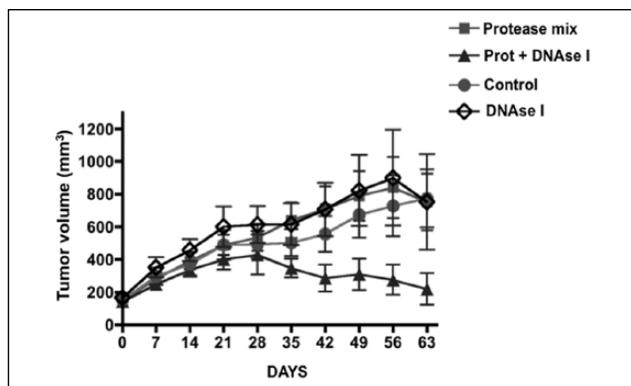
Figure 5. Antitumor effect of the enzymatic treatment in athymic mice. The graph shows the effect on the tumor size in tumor-bearing mice treated with the enzyme mix (papain: 12.5 mg/kg + trypsin: 5 mg/kg + chymotrypsin: 5 mg/kg + DNAse I: 2.3 mg/kg). Statistically significant differences were found between the untreated group versus the protease mix plus DNAse I mix ($P = 0.011$) group but not against the groups treated with either DNAse I alone or protease mix alone (Student's *t* test).

On the other hand, the biological meaning of serum protein reduction in rats after the enzyme treatment is unknown. Nevertheless, the fact that these enzymes degrade DNA and proteins *in vitro* and *in vivo* suggest that the antitumor effect could be related to depletion of circulating DNA. The fact that treatment of mice with proteases only did not exhibit antitumor effects further suggests that circulating DNA could be mostly responsible for the phenomenon. Nevertheless, our findings are solely hypothesis generating because no experimental data are provided to demonstrate that, indeed, the depletion of circulating DNA led to tumor reduction.

A number of studies have demonstrated the ability of extracellular DNA from cell culture supernatant, serum/plasma, or apoptotic bodies to horizontally drive transformation and tumorigenesis. Using supernatant of cultured SW480 cells Anker et al[11] showed NIH3T3 transformation associated with mutant *K-Ras* transfer. García-Olmo et al[12] observed cell transformation and tumorigenesis of NIH3T3 "passively" transfected with human plasma of colon cancer patients. In addition, they showed that plasma from healthy individuals was unable to do so. Similar results have been obtained using apoptotic bodies as a source of exogenous DNA. Bergsmedh et al[17] used *H-ras/* human *c-myc*-transfected rat fibroblasts as donor and mouse embryonic fibroblasts as recipient cells, whereas Gaiffe et al[18] demonstrated cell transformation using human papillomavirus–positive cervical cancer cells and human mesenchymal cells as source and recipient cells, respectively. Taken together, these data suggest that depleting the oncogenic DNA from circulation could have antitumor effects.

To our knowledge, this is the first study evaluating the systemic administration of this enzymatic combination under the rationale that depleting circulating DNA could have antitumor effects. Very early reports exist on the use of intravenous bovine crystalline pancreatic desoxyribonuclease for treating patients with meningeal and pulmonary tuberculosis, lung abscesses, and other bacterial suppurative conditions. Of note, treatment was well tolerated, even using doses ranging in millions of units.[19,20] More recently, recombinant DNAse I (rhDNAse) has also been tested. Patients received rhDNAse at 25 or 125 µg/kg of rhDNAse or placebo in a schedule comprising a single intravenous dose followed by 10 subcutaneous doses. No adverse events were registered, and serum concentrations of rhDNAse (between 40 and 100 ng/mL) that have enzymatic activity were achieved by substantial time periods; nevertheless, no direct measurements of extracellular DNA were performed in either of these studies.[21] Systemic DNAse has also been evaluated in a number of experimental systems. Alcazar-Leyva et al[22] showed that DNAse inhibits cell proliferation *in vitro*, whereas *in vivo*, its systemic administration slows the course of lymphatic leukemia in AKR mice[23] and also prevents liver metastases in cutaneously transplanted tumor cells in mice.[24]

Patutina et al[25] reported that daily administration of RNAse and DNAse, either alone or in combination, reduces the pathologically increased level of extracellular DNA and increased nuclease activity of the blood plasma of tumor-bearing mice back to the level of healthy animals. This decrease in circulating DNA, which was increased in tumor-bearing animals, was associated with reduced formation of metastases in 2 murine models using Lewis lung carcinoma and HA-1 hepatoma xenografted in the thighs as well as in a model of intravenously injected Lewis lung carcinoma cells. The clinical use of proteases, specifically pancreatic enzymes, has been pursued for many years. Between 1900 and 1910, at least 13 publications reported on the efficacy of this treatment in a number of solid tumors.[26] Currently, active trypsin, chymotrypsin, and other proteases are also components of the commercially available enzyme mixtures Wobe-Mugos E and Phlogenzym (Mucos Pharma GmbH, Geretsried, Germany), which have been tested by the oral route in cancer patients for relief of chemotherapy- and radiation-related symptoms. There are no studies evaluating possible therapeutic activities of proteases by intravenous administration; however, it has been shown that the subcutaneous administration of an enzyme mixture containing trypsinogen, amylase, chymotrypsinogen, and traces of the active chymotrypsin leads to a remarkable increase in survival rate of female C57B16 mice injected subcutaneously with B16F10 cells.[27] Likewise, a mixture of amylase and trypsinogen led to significantly slow growth of methyl-cholantrene-induced tumors in mice.[28] The protease mix used in our work—trypsin, chemotrypsin, and papain—was

given by intrarectal administration, demonstrating growth inhibition at the primary tumor, antimetastatic effect, and an increase in survival in C57B16 mice with subcutaneous implantation of B16F10 cells.[25,27,28] In this regard, our results in the mice are noticeable because 40% of them had complete, histopathologically evaluated eradication of the tumor at the expense of no toxicity, as clinically and pathologically observed, suggesting that this treatment can be feasible; but of course, much more work is needed before the treatment can be clinically tested.

All together, our results support previous findings on the antitumor effects of the combination of DNAse I and mix of proteases; however, it is to be noted that in our model, neither DNAse I nor the protease mix alone exhibited growth inhibition, despite the fact that the doses of DNAse I and proteases used were similar to that in previous work.[25,27,28] This result may be related to the experimental model itself (nude mice injected with human cancer cells), which is different from that used in previous reports, or alternatively, because of the administration route we used: where possible, the plasma concentrations achieved in our model were higher.

Regarding circulating DNA, although it seems clear that tumor cells in cancer patients shed DNA into the circulation and that this correlates with the extent of disease,[29] and this circulating DNA has neoplastic characteristics[30] and carries the genetic alterations found in the primary tumor,[31] the biological meaning of circulating DNA is yet to be understood. On this basis, more studies are needed to confirm that tumor-shed DNA indeed drives tumor progression in patients. In addition, it is important to understand how DNA travels in the circulation to achieve its efficient depletion by pharmacological or other means. On the other hand, the use of proteases as cancer treatment seems counterintuitive to current knowledge that metalloproteinases and other proteases are overexpressed in cancer and seem to be critical for tumor invasion and metastases.[32] However, this may not hold true in general because phase III clinical trials of matrix metalloproteinase inhibitors marimastat, prinomastat, and batimastat administered alone or in combination with chemotherapy in patients with advanced cancers (lung, prostate, pancreas, brain, gastrointestinal tract) failed to show clinical efficacy.[33] In fact, the expression of certain matrix metalloproteinases, either at the primary or the metastatic site, provides a beneficial and protective effect in multiple stages of cancer progression.[34] In this sense, it has been hypothesized that trypsin may have tumor suppressive effects because it is silenced by promoter methylation,[35] and several studies showed that high-grade tumors express trypsinogen, whereas low-grade tumors harbor lower expression.[36-38] Furthermore, other cysteine proteases, fastuosain and bromelain, were shown to have antitumor effects in a B16F10 model of murine melanoma,[39] and other works demonstrate that *in vitro* treatment of several human cancer cell lines with pancreatic enzymes suppresses the epithelial-mesenchymal transition and promotes cell differentiation.[40]

The field of the biological meaning of circulating DNA as a therapeutic target is not new. However, studies on it are very scarce, and the results of this work must be seen with caution because we did not demonstrate that the treated mice had a decrease in circulating DNA or changes in serum protein levels. Nevertheless, that reduction occurred can be suggested by the results obtained in rats, which showed a decrease in both parameters. Again, the major weakness of the study is that it is not mechanistic; hence, at this time, we still do not know how the antitumor effect occurs. In summary, our results further support the concept that depleting the circulating DNA by the use of DNAse I and a protease mix containing trypsin, chymotrypsin, and papain, which are needed to help DNAse I digest DNA, may have a role in cancer treatment. Nevertheless, it is necessary first to understand how circulating DNA drives tumor progression and the pharmacokinetics and pharmacodynamics of the enzymatic combination resulting in serum DNA depletion. To further complicate the picture, the effects of systemic proteases most likely heavily influence tumor biology by altering the balance between protumoral and antitumoral cytokines, chemokines, growth factors, and other proteins.

Declaration of Conflicting Interests

Funding

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