The Origin and Mechanism of Circulating DNA

Article *in* Annals of the New York Academy of Sciences · May 2000

DOI: 10.1111/j.1749-6632.2000.tb06608.x · Source: PubMed

The Origin and Mechanism of Circulating DNA

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FROM WHERE DOES CIRCULATING DNA COME?

Although it is evident that DNA circulates freely in blood plasma both in disease and in health, the source of this DNA remains enigmatic. It can be presumed that circulating DNA in healthy subjects derives from lymphocytes or other nucleated cells. Yet, it is not known why cancer patients have such large quantities of plasma DNA. There is no doubt that a proportion seems to originate from nucleated blood cells since wild-type DNA has been detected in the plasma of all cancer patients studied as well as in that of healthy controls. However, a substantial proportion of plasma DNA in cancer patients derives from tumor cells. This latter concept is supported by both quantitative and qualitative observations. Plasma DNA levels are not only greater in cancer patients than in normal subjects,[1–5] but also correlate inversely with outcome and tend to fall with effective treatment.[1,5] In addition, the ability to detect LOH in plasma DNA[6–13] suggests that tumor-related DNA is the predominant subtype in at least some cancer patients.

What is responsible for the presence of tumor DNA shed in the bloodstream? It could be due to lysis of circulating cancer cells or of micrometastases, to DNA leakage resulting from tumor necrosis or apoptosis, or to a new mechanism of active release.

LYSIS OF CIRCULATING CANCER CELLS OR OF MICROMETASTASES

The most common hypothesis advanced for circulating DNA in the plasma of cancer patients is that it is due to the lysis of circulating cancer cells or micrometastases shed by the tumor. This is clearly not the case since there are not enough circulating cells to justify the amount of DNA found in the plasma. Sorenson[14] calculated that, in relation to the amount of DNA that he found in the plasma of pancreatic cancer patients, there would have to be 1000 cancer cells per mL, which is far more than has ever been found. Using another DNA extraction procedure (Boehringer columns)[12] that yields 10 times more DNA than the method used by Sorenson (Qiagen Kit), it would have to be assumed that 10,000 tumor cells per mL are circulating in the blood-

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stream. Now, in the case of colorectal patients, no ras mutations have been found in the cells of the Ficoll layer where micrometastatic cells should be found. Moreover, the same mononuclear cells that should include circulating tumor cells, if there are any, were used as control of normality in all microsatellite analyses.[6–13]

TUMOR NECROSIS

Tumor necrosis has been postulated to explain plasma DNA of cancer patients since higher amounts of DNA were found in the plasma of patients with large tumors or with advanced diseases with metastases,[2–6,10,15] although it should be reminded that plasma tumor DNA is also found in early stages.[8,9,11–13,16–18] An argument against necrosis is that the plasma DNA levels decreased up to 90% after radiation therapy,[1] whereas one might expect an initial plasma DNA surge following radiotherapy if necrosis was the predominant pathway for DNA release.

APOPTOSIS

Apoptosis has been advanced as the origin of circulating DNA on the basis of several observations, the main one being that plasma or serum DNA often presents a ladder pattern after electrophoresis that is reminiscent of the pattern shown by apoptotic cells.[3,5,19] Giacona *et al*.[19] have evaluated the plasma DNA by gel electrophoresis and measured the variation in length of soluble DNA fragments by electron microscopy in plasma from three patients with pancreatic cancer and from three healthy controls. Nick-translated DNA isolated from plasma and subjected to gel electrophoresis and electron microscopy displays different band patterns depending on whether the plasma originates from a normal subject or a cancer patient. Small excesses of DNA at approximately 63, 126, 189, 252, and 315 nm, corresponding to small multiples of lengths associated with nucleosomes, were more prominent in the cancer patient plasma than in the healthy control plasma. These authors conclude that a significant proportion of plasma DNA derives from apoptosis of neoplastic cells. Using the same arguments, Fournié came to the same conclusions.[5] He also based his assumption on the correlation that he observed between the quantity of serum DNA and the activities of neuron-specific enolase and lactate dehydrogenase, which are considered as cell death markers in cancer patients.[5] Let us note, however, that apoptosis is a mechanism supposedly lost by proliferating cancer cells, and great efforts are made to restore programmed cell death in malignant cells. Moreover, the paradigm of apoptosis implies that cell death products, particularly those containing DNA such as apoptotic bodies, are cleared *in situ* by epithelial cells and macrophages without any inflammatory effect.[20,21]

SPONTANEOUS AND ACTIVE RELEASE OF DNA

As a fourth possibility, it may be hypothesized that the tumor actively releases DNA into the bloodstream. If one cannot affirm that the circulating tumor DNA proceeds by the same mechanism as observed *in vitro* where lymphocytes or whole

TABLE 1. Experimental results

	Amount of DNA (mg/culture)		Specific activity (cpm/mg)	
Time of labeling	Cellular	Extracellular	Cellular	Extracellular
Experiment 1	627	13.5	187	235
Experiment 2	644	13	277	2700

NOTE—Experiment 1: first incubation (3 h) in the presence of ^{3}H-TdR followed by a second incubation (4 h) without label. Experiment 2: first incubation (16 h) in the presence of ^{3}H-TdR followed by a second incubation (4 h) without label.

organs spontaneously release DNA,[22–27] it remains possible and plausible that both phenomena are related. It is worth recalling some data on this subject.

Cells (lymphocytes) or even whole organs in culture (frog auricles) spontaneously release a nucleoprotein complex within a homeostatic system in which the newly synthesized DNA is preferentially released.[23–26] The possibility that this release could have been a consequence of cell death has been ruled out by the following experiments: (a) the same amount of DNA was found in the medium whether the incubation lasted 2, 4, or as long as 72 h (FIG. 1), instead of an increasing amount with time, which would be expected if cells were dying; (b) cell death had no effect on the amount of extracellular DNA (frog auricles were cut in pieces and lymphocytes were deprived of nutrients or heated); (c) when the cells or organs were resuspended in new medium several times in a row, a similar amount of extracellular DNA was isolated from each of the successive supernatants; in contrast, if after removal the cells or organs were put back in their original medium, no increase in the amount of extracellular DNA was observed (FIG. 1), suggesting a precise homeostatic mechanism independent of any mechanical effect; (d) the specific activity of the released DNA was different (higher) than that of the cellular DNA, suggesting a preferential release of newly synthesized DNA (TABLE 1); and (e) the cells and organs that had excreted DNA kept their functional integrity, as shown for the cells by their fully maintained capacity to respond to stimulation and for the auricles by their maintained rhythm of beats per minute. All these characteristics argue against apoptosis.

Other experiments on this phenomenon of DNA excretion have been performed on stimulated lymphocytes. Typical results can be seen in FIGURE 2, where lymphocytes have been stimulated with tuberculin for five days. About 40% of the cells undergo blast transformations and synthesize DNA. However, less than 10% of these lymphocytes actually undergo mitosis, while up to 90% of the newly synthesized DNA is excreted into the medium. Between day 3 and day 5 of culture, cell mortality never exceeds 11%, thus excluding cell death as the origin of the extracellular DNA found. These kinds of results have been obtained by several teams.[28–35] Amongst them, Rogers and colleagues have found that, after phytohemagglutinin activation, lymphocytes selectively replicate several copies of a limited portion of their genome, copies that are then excreted into the culture medium.[29,30] A portion of the DNA released by PHA-stimulated human lymphoblasts becomes bound to the plasma membranes of the cells and is actively capped. Capping is accompanied by shape change,

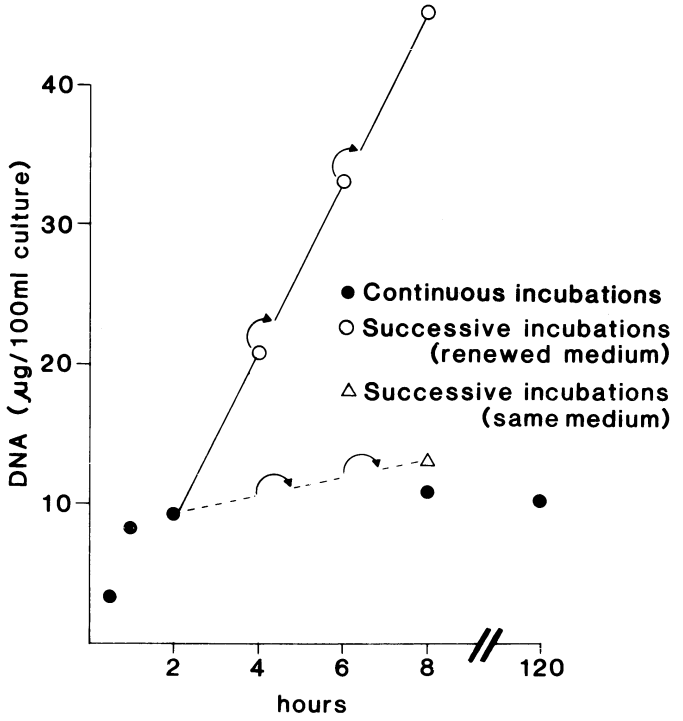

FIGURE 1. Amount of DNA extracted[24] from the supernatant of lymphocyte cultures. A culture of lymphocytes (around 10^6 cells/mL) was divided into three parts of 200 mL. These cultures were submitted to different incubations: (a) the lymphocytes were cultured for various periods up to 120 h in the same medium; (b) the lymphocytes were submitted to four successive incubations of 2 h; the lymphocytes were centrifuged at the end of each incubation period, the supernatant was removed, and the lymphocytes were put back in the same medium; and (c) the lymphocytes were centrifuged at the end of each incubation period and put back in a new medium.

and caps are localized to uropods.[30] The marked difference between released DNA and cell DNA indicates that this extracellular DNA is not derived from dying cells. That such a mechanism might occur *in vivo* has been demonstrated in mice in which a release of DNA in plasma has been observed after injection of bacterial lipopolysaccharides, which has a mitogenic effect comparable to PHA.[31] We have observed that cancer cells in culture release more DNA than normal cells, for instance, cells from leukemic patients compared to lymphocytes from healthy donors (unpublished results). It is thus not surprising, if the circulating DNA is due to an active release, to find more plasma DNA in cancer patients than in healthy controls. Further work is needed to determine the relation between apoptosis and/or active release and the presence of plasma DNA.

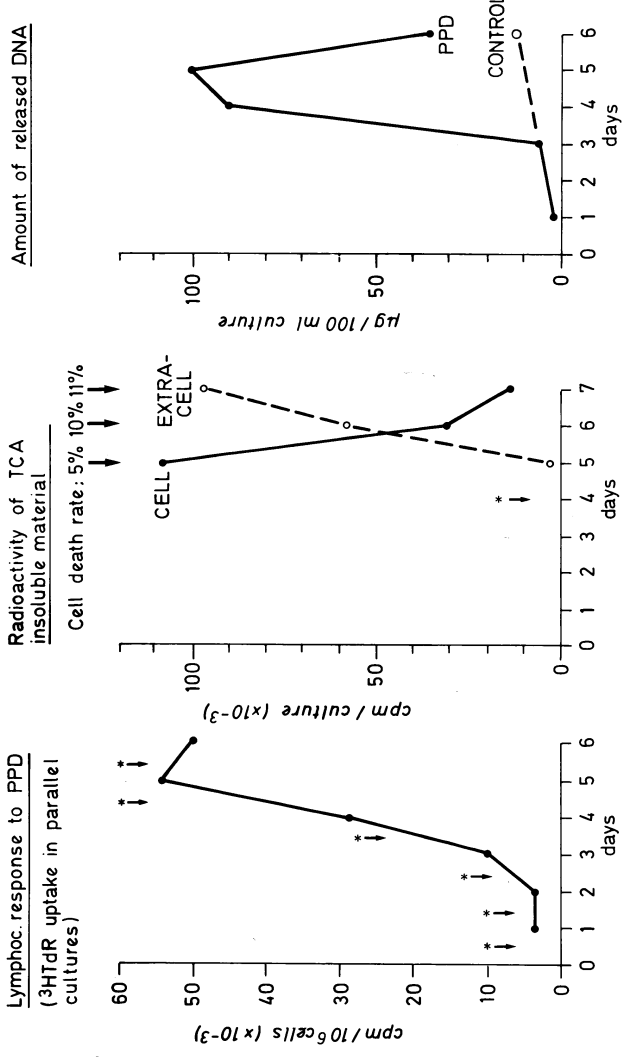

FIGURE 2. DNA released by PPD-stimulated lymphocytes. (Left) Lymphocyte response to PPD (^{3}H-thymidine uptake in parallel cultures)—abscissa: days of incubation; ordinate: radioactivity (cpm/10^6 cells). (Center) Radioactivity of DNA (TCA insoluble material) found in cells and in supernatant—abscissa: days of incubation; ordinate: radioactivity (cpm/culture). (Right) Amount of released DNA—abscissa: days of incubation; ordinate: amount of released DNA (mg/100 mL culture).

WHY?

The function of this extracellular DNA, whether it is due to apoptosis or active release, is unknown. It may have a physiological and/or pathological role. For instance, DNA has been shown to spontaneously pass from prokaryotic cells to eukaryotic cells,[36–38] where it may be transcribed. The passage of DNA from eukaryote to eukaryote cells may also occur.[39–41] In the case of DNA transfer between bacteria and higher organisms, the case of crown gall, a plant cancer, is particularly interesting. It was found a long time ago that the DNA spontaneously released by the bacteria *Agrobacterium tumefaciens*,[42,43] which was later found to be a plasmid,[44] induced the tumor. Now, crown gall can give rise to metastases. Plant cells are held together by the network of their pectocellulosic walls, which prevent the migration of transformed cells. Thus, in the case of plants, metastasization is probably due to circulating DNA. This might also occur in some cases in mammals, as shown by the malignant transformation of donor cells observed sometimes after a bone marrow graft.[45]

The hypothesis that circulating tumor DNA in the plasma might play a role in metastasis has been advanced. It has been shown *in vitro* that DNA released from SW480 cells has a high transforming activity. Indeed, when crude SW480 cell supernatant is given, without any adjunction, to NIH3T3 mouse cells, transformed foci appear as numerous as those occurring after a transfection with a cloned ras gene administered as a calcium precipitate.[46] In an *in vivo* study, healthy rats having received plasma from a tumor-bearing rat showed the presence of the tumor marker gene in their lung DNA.[47]

On another line, some studies have suggested that extracellular DNA could play a role in the impairment of natural killer (NK) activity observed in cancer patients: plasma from patients with metastatic cancer characterized by increased concentrations of DNA significantly inhibited the NK activity of normal lymphocytes.[5]

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