

DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells¹

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ABSTRACT

Increased levels of DNA fragments have frequently been found in the blood plasma of cancer patients. Published data suggest that only a fraction of the DNA in blood plasma is derived from cancer cells. However, it is not known how much of the circulating DNA is from cancer or from noncancer cells. By quantitative methylation-specific PCR of the promoter region of the *CDKN2A* tumor suppressor gene, we were able to quantify the fraction of plasma DNA derived from tumor cells. In the plasma samples of 30 unselected cancer patients, we detected quantities of tumor DNA from only 3% to as much as 93% of total circulating DNA. We investigated possible origins of nontumor DNA in the plasma and demonstrate here a contribution of T-cell DNA in a few cases only. To investigate the possibility that plasma DNA originates from apoptotic or necrotic cells, we performed studies with apoptotic (staurosporine) and necrotic (staurosporine plus oligomycin) cells *in vitro* and with mice after induction of apoptotic (anti-CD95) or necrotic (acetaminophen) liver injury. Increasing amounts of DNA were found to be released in the supernatants of cells and in the blood plasma samples of treated animals. A clear discrimination of apoptotic and necrotic plasma DNA was possible by gel electrophoresis. The same characteristic patterns of DNA fragments could be identified in plasma derived from different cancer patients. The data are consistent with the possibility that apoptotic and necrotic cells are a major source for plasma DNA in cancer patients.

INTRODUCTION

It is known that double-stranded DNA fragments frequently occur in considerable quantities in the serum or plasma of cancer patients (1, 2). The quantitation of this free DNA in the serum of patients with various types of cancer and healthy individuals showed that the DNA concentration in the normal controls had a mean of 13 ng/ml, whereas in the cancer patients the mean was 180 ng/ml. Although no correlation was found between circulating DNA levels and the size or location or the primary tumor, significantly higher DNA levels were found in the serum of patients with metastases (2). Other studies performed with lung cancer patients found plasma DNA levels to be higher in patients with advanced disease (3, 4). Investigations on the characteristics of the DNA found in the plasma of cancer patients showed that an important part of the DNA originates from the tumor cells (5). Furthermore, the presence of oncogene or tumor suppressor gene mutations that characterize DNA in tumor cells were detected in plasma DNA. For example, the *K-ras* mutations in the DNA derived from pancreatic tumors were also found in the circulating DNA of the same patients (6), just as the same microsatellite alterations, detected in head and neck carcinomas, small cell lung carcinomas, or renal carcinomas, could be determined in patients' plasma DNA (7–9). Furthermore, tumor-specific epigenetic alterations such as the hypermethylation of sequences in the promoters of tumor suppressor genes

could frequently be identified in the plasma DNA of carcinoma patients (10–12). These and similar findings indicate that a certain percentage of circulating DNA originates from degenerating tumor cells. It has therefore been proposed that analyses of plasma DNA could be useful for prognostic purposes or for early diagnosis to detect, *e.g.*, subclinical disease recurrence in disease-free patients (13). An analysis might also constitute a tool to follow the development of tumors by monitoring genetic changes in the circulating DNA of cancer patients. However, the amounts of DNA in plasma vary widely even in clinically similar situations (1, 2). Moreover, reports quite commonly include cases where mutated genes could be detected in tumor tissue but not in circulating DNA (13), suggesting that not all of the plasma DNA originates from tumor cells, whereas other studies suggest that tumor DNA is the predominant subtype in at least some cancer patients because tumor-specific loss of heterozygosity was detected in plasma DNA (7–9). In fact, the mechanism of how DNA is released into blood circulation is unknown, though there are many hypotheses, such as tumor cell apoptosis or necrosis or active release of DNA (13).

Because the issue of circulating DNA is not only of clinical relevance but also of considerable biological interest, we decided to investigate the origin of DNA in the plasma samples of 30 cancer patients of different tumor types. Using highly sensitive methods of quantitative PCR, we determined the fraction of DNA in the circulation that originates from tumor cells and from nontumor cells. We also sought for evidence showing that at least some of the DNA could be released from apoptotic or necrotic cells, a conclusion that we support by studies using cultured cells and mice with *in vivo*-induced apoptosis and necrosis.

MATERIALS AND METHODS

Plasma Sample Collection and DNA Extraction. Blood samples were withdrawn from a peripheral vein and placed in EDTA-containing tubes from a total of 30 unselected informed cancer patients diagnosed at the Department of Oncology at the municipal hospital. Similarly, blood was drawn from 20 healthy donor volunteers. The study was approved by the Ethics Committee of the University of Konstanz. Plasma was immediately separated from blood cells by centrifugation at $3000 \times g$ for 20 min. Tumor tissue from patients was collected at surgery. DNA was extracted from blood plasma and tumor tissue using the QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol (11, 14) or the QIAamp Tissue Kit, respectively (11).

Quantitation of Total Plasma DNA. The amounts of plasma DNA were determined by competitive PCR according to the method of Diviacco *et al.* (15), using the lamin B2 locus as a typical example for a single copy gene. The competitor molecule carrying a 20-bp insert was obtained directly from two amplification products by the overlap extension method (15). Quantitation of competitive templates was obtained by OD₂₆₀ measurement. For quantitation, a fixed amount of plasma DNA was mixed with increasing amounts of the competitor template. For competitive PCR, two additional primers (Q-EF: 5'-TCCAATGATTTGTAATATAC-3' and Q-ER: 5'-ATCTTTCTTAGA-CATCCGCTT-3') were designed. After PCR amplification (40 cycles: 94°C, 1 min; 52°C, 1 min; 72°C, 1 min) and PAGE, two products of 153 and 173 bp were evident, corresponding to genomic and competitor templates, respectively. The ratios of the amplified products precisely reflect the initial concen-

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tration of genomic DNA *versus* that of the added competitor. Quantitation of competitor and genomic bands was obtained by densitometric scanning of the ethidium bromide-stained gel (15). The results obtained by means of competitive PCR were confirmed by quantitation with the Control Kit DNA in the LightCycler System (Roche Diagnostics). For that purpose we used the LightCycler Control Kit DNA to amplify a 110-bp fragment of the human β -globin gene. The amplicon was detected by fluorescence using a specific pair of hybridization probes (LC-Red 640). As standards we used serially diluted genomic DNA of the kit. After completion of PCR, the LightCycler software calculated the copy number of target molecules by plotting logarithm of fluorescence *versus* cycle number and setting a baseline *x*-axis. The concentrations of the samples were extrapolated from the standard curve by the LightCycler software.

T Cells. The presence of T-cell DNA in plasma samples was examined by PCR amplification of a region of the T-cell receptor β chain, which exhibits a somatic rearrangement by VDJ recombination (16). For amplification, a mix of sense primers (*V β 5*, *V β 6*) and one antisense primer (*J β 1i*) were used (16). This leads to the amplification of DNA fragments with defined sizes: 907, 767, and 155 bp, depending on the rearranged β -segment. DNA from Jurkat T cells, HeLa cells, and human lymphocytes was included as internal controls with each run. As an additional control, we amplified plasma DNA with primers specific for the germline configuration of the T-cell receptor using the primers G1F: 5'-AATGATTCAACTCTACGGGA-3' (sense) and G1R: 5'-TGAGTCTCCACTTGTGAG-3' (antisense), resulting in a product of 250 bp. One hundred ng of purified plasma DNA samples were used in each PCR assay. PCR products were analyzed by 6% PAGE and ethidium bromide staining.

Hypermethylation of *CDKN2A*. Detection of hypermethylated CpG islands in the promoter region of the *CDKN2A* tumor suppressor gene was carried out by methylation-specific PCR as previously described (17). Bisulfite treatment of tumor or plasma DNA was carried out using the CpGenome DNA modification kit (Intergen). As a positive control, normal human dermal fibroblast cell DNA was methylated *in vitro* using the CpG methylase (New England BioLabs). PCR products were analyzed after electrophoresis on 6% polyacrylamide gels.

Quantitation of Hypermethylated *CDKN2A*. The ratio between unmethylated and hypermethylated *CDKN2A* alleles in plasma of cancer patients was analyzed using methylation-specific quantification in the LightCycler system (Roche Diagnostics). For amplification of unmethylated DNA, we used primers according to Herman *et al.* (17). For the methylated *CDKN2A*, the following set of PCR primers was used: 5'-GGTGGGGCGGATCGC-3' (sense) and 5'-CCGAACCGCGACCGTAA-3' (antisense). Two sets of LC-Red 640-labeled, methylation-specific hybridization probes were used for detection of real-time PCR products in the LightCycler reaction: for the unmethylated reaction 5'-CTCCCCACCACCCACTACTCT-3' (p16N FL) and 5'-CCCCTCTCCACAACCACCAACAC-3' (p16N LC) and for the methylated reaction 5'-CCGCCGCCGCTACTACTCT-3' (p16M FL) and 5'-CCTCTCCGCAACCGCGAAC-3' (p16M LC). PCR (40 cycles of denaturation for 10 s at 95°C, annealing for 10 s at 69°C, and extension for 10 s at 72°C) was performed with the LightCycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics). A fixed volume of 4 μ l deaminated plasma DNA was applied in each PCR assay. *In vitro* methylated DNA and DNA from normal human lymphocytes were used as standard DNA for the quantitation of methylated and unmethylated *CDKN2A*, respectively. The efficiency of the *in vitro* methylation of the standard DNA was analyzed by real-time PCR before quantifications: in the unmethylated *CDKN2A* reaction no product was visible using this *in vitro* methylated DNA as template. For all of the assays the same standard DNA was used. The methylation-specific measurements of plasma DNA were all performed in duplicates. The difference between the samples in each LightCycler assay was about \pm 10% for both unmethylated and methylated DNA.

Endothelial Cells. To investigate the presence of endothelial cell DNA in the plasma, a methylation-specific PCR-based method was established. After bisulfite treatment of purified plasma DNA (CpGenome Modification Kit; Intergen), a methylation-specific PCR in the promoter region of the *selectin E* (*SELE*) gene was carried out. Two different sets of primers were used to distinguish the unmethylated from the hypermethylated *SELE* promoter: unmethylated: 5'-ATTTTAAGTATTGTGGATATTTTG-3' (sense) and 5'-CAAAAACAACCTAAACTACTTCA-3' (antisense); hypermethylated: 5'-

TTTAAGTATCGTGGATATTTTCG-3' (sense) and 5'-AAAAACAACCTAA-CACTACTTTCG-3' (antisense). The sequence-specific primers cover three CpG islands, two of which are positioned at the 3' end of the primers. Thirty-five PCR cycles were performed, with each cycle consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. DNA of HeLa cells and of HUVECs³ served as negative and positive controls, respectively. PCR products (151 bp) were visualized after electrophoresis on 6% polyacrylamide gels and ethidium bromide staining.

Induction of Apoptosis and Necrosis *in Vitro*. Jurkat T cells in serum-free medium without glucose were induced to undergo necrosis (with 2.5 μ M oligomycin plus 1.2 μ M staurosporine) or apoptosis (with 1.2 μ M staurosporine) as previously described (18). At different times after the addition of drugs, the supernatant was separated from the cells by centrifugation. The DNA of the supernatants was purified (QIAamp blood kit; Qiagen) and quantified by competitive PCR. Apoptotic and necrotic cell death were confirmed by monitoring the cleavage of the nuclear SAF-A in Western blot analyses. Only during apoptotic, but not necrotic cell death, is SAF-A cleaved by caspase-3 (19, 20).

Induction of Apoptotic and Necrotic Liver Injury in Mice. Specific pathogen-free male BALB/c mice (~25 g, from the in-house animal breeding station of the University of Konstanz) were maintained under controlled conditions (22°C and 55% humidity, constant 12-h day/night cycle) and fed a standard laboratory chow. All animals received humane care in accordance with the NIH guidelines as well as with the legal requirements in Germany. Mice were starved overnight before the onset of experiments. To induce hepatocyte apoptosis, activating anti-CD95 antibody (α CD95, clone Jo-2, 2 μ g per animal; PharMingen) was injected *i.v.* in a volume of 300 μ l endotoxin-free saline supplemented with 0.1% human serum albumin (21, 22). Hepatocyte necrosis was induced by *i.p.* treatment of mice with acetaminophen (250 mg/kg in 300 μ l endotoxin-free saline; EGA, Steinheim, Germany; Ref. 20). At the time points indicated, mice were killed by *i.v.* injection of 150 mg/kg pentobarbital plus 1.2 mg/kg sodium citrate as an anticoagulant. Blood was withdrawn by cardiac puncture and centrifuged (5 min, 14,000 \times g, 4°C) to obtain plasma. The extent of liver damage was assessed by measuring plasma ALT activity with an EPOS 5060 analyzer (Netheler & Hintz, Hamburg, Germany) as previously described (23). DNA was extracted from murine plasma using a DNA extraction kit (QIAamp blood kit; Qiagen) and quantified by real-time PCR (LightCycler; Roche Diagnostics). The region of amplification was part of exon 3 of the murine α -actin gene. The following primers were used for amplification: 5'-TGAACATGGCATCATCACC-3' and 5'-CTG-GATAGCCACATACATG-3', resulting in a product of 199 bp. Real-time quantitation was carried out by the SYBR-Green reaction mix (Roche Diagnostics). A fixed amount of 4 μ l of eluted DNA was used in each PCR reaction. Forty PCR cycles were performed, with each cycle consisting of 1 s at 95°C, 5 s at 55°C, and 10 s at 72°C.

Analysis of Plasma DNA Fragments. DNA of human or murine plasma was extracted using a DNA extraction kit (QIAamp blood kit; Qiagen) and separated on 6% polyacrylamide gels (patients' plasma DNA samples) or 1.5% agarose gels (murine plasma DNA samples). Fragments were then visualized after ethidium bromide staining.

RESULTS

Quantitations. We determined the concentration of DNA in plasma samples of 30 unselected cancer patients (Table 1). For that purpose, we used primers corresponding to a subtelomeric single-copy region of chromosome 19 in the quantitative PCR procedure of Diviacco *et al.* (15). The data obtained by quantitative PCR were verified by real-time quantitation in a LightCycler instrument (Roche Diagnostics). The difference between the results obtained by these two methods was \pm 13%. All quantitations were performed in duplicate, with a reproducibility of \pm 12.5% in the competitive assays and \pm 10% in the LightCycler assays. The results of quantitation revealed a wide spectrum of DNA concentrations in the plasma of cancer patients, between 10 and 1200 ng/ml, with a mean of 219 ng/ml

³ The abbreviations used are: HUVEC, human vascular endothelial cell; SAF, scaffold attachment factor; ALT, alanine aminotransferase.

Table 1 Plasma DNA: diagnoses; quantitations; presence of T-cell DNA; and hypermethylation of CDKN2A promoter

Patient	Cancer	DNA (ng/ml)	T cells ^a	CDKN2A ^b
C1	Colorectal	167	-	-
C2	Angiosarcoma	19	ND ^c	+
C3	NSCLC	100	-	ND
C4	Breast	66	-	-
C5	Breast	134	-	ND
C6	SCLC	33	-	ND
C7	Breast	170	-	ND
C8	Breast	83	-	+
C9	Ewing's sarcoma	60	-	+
C10	Breast	20	ND	+
C11	NSCLC	84	ND	-
C12	Head and neck	10	ND	+
C13	Melanoma	20	ND	+
C14	Pancreas	1200	+	-
C15	Colorectal	67	-	-
C16	Colorectal	25	ND	ND
C17	Colorectal	50	ND	-
C18	Breast	38	ND	-
C19	Breast	90	-	-
C20	Pancreas	48	-	+
C21	HCC	84	-	-
C22	Urothel	168	-	-
C23	Breast	360	-	+
T1	NSCLC	550	-	+
T2	Colorectal	555	-	+
T3	Esophagus	833	-	+
T5	Stomach	500	+	-
T6	Colorectal	980	-	-
T7	Colorectal	38	ND	-
T8	Colorectal	34	ND	-

^a + or -, presence or absence, respectively, of T-cell DNA in plasma samples.

^b +, hypermethylation of CDKN2A gene promoter; -, no hypermethylation detected.

^c ND, not done; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; HCC, hepatocellular carcinoma.

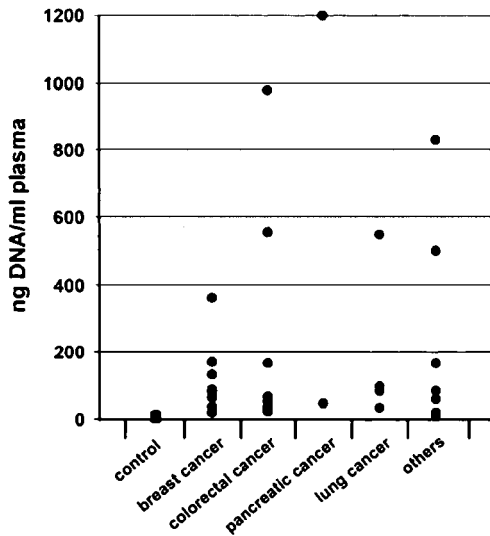


Fig. 1. DNA levels in the plasma of tumor groups (24 patients) and 14 healthy individuals, obtained by competitive PCR. The values represent the average of duplicate determinations. Control, 14 healthy individuals. For patients, see Table 1.

(Fig. 1). We observed no clear correlation between the concentrations of DNA in the plasma and the clinical situation such as diagnosis, tumor staging, or therapy, *e.g.*, advanced pancreatic adenocarcinoma was diagnosed in patients C20 and C14, yet patient C20 had the relatively low concentration of 48 ng DNA/ml plasma, whereas patient C14 had the highest plasma DNA concentration (1200 ng/ml; Fig. 1; Table 1). In fact, some of the values in the group of cancer patients were not higher than the DNA concentrations determined in the plasma of two persons in the control group (mean, 3.7 ng DNA/ml plasma; highest values, 10–15 ng/ml). However, most of the control cases had <2 ng/ml DNA in their plasma (Fig. 1), which is at the limit

of sensitivity of the competitive assays. Thus, we conclude that elevated levels of circulating DNA appear to be a characteristic feature of most, but not all of the carcinomas.

T-Cell DNA. We considered the possibility that degenerating tumor-infiltrating T lymphocytes contribute to the DNA levels in blood plasma (24). We, therefore, prepared two sets of PCR primer pairs: one pair of primers, originally designed for the detection of different clonal rearrangements, identifies rearranged T-cell receptor β chain genes in mature T cells (16), whereas a second newly designed primer pair determines the germline configuration found in all of the non-T cells (Fig. 2A). In control experiments, the T-cell-specific primers amplified the clonal rearranged genomic sequence in the Jurkat T-cell line (Fig. 2B, Lane J) and in WBCs (Fig. 2B, Lane B) but gave negative results with HeLa DNA (Fig. 2B, Lane H). Conversely, primers specific for the germline configuration amplified the expected sequence in HeLa cell DNA and WBC DNA, but not in Jurkat cell DNA (Fig. 2C, Lanes H, J, and B). Using these primers, we detected mature T-cell DNA sequences in the plasma DNA samples C14 (pancreatic adenocarcinoma, 1200 ng DNA/ml plasma; Fig. 2B) and T5 (stomach cancer, 500 ng/ml), whereas all of the other plasma DNA samples examined were devoid of detectable T-cell DNA (Table 1). All of the plasma samples of cancer patients examined gave positive results with the germline PCR (Fig. 2C). This result leads to the conclusion that DNA in the circulation of cancer patients does not very frequently contain T-lymphocyte DNA.

Origin of Plasma DNA. The primary aim of the present study was to gain insight into the origin of circulating DNA in cancer patients. As we have just seen, lysis of T cells does usually not contribute to plasma DNA, and the question arises how much of the plasma DNA originates from tumor cells. For that purpose, we determined the

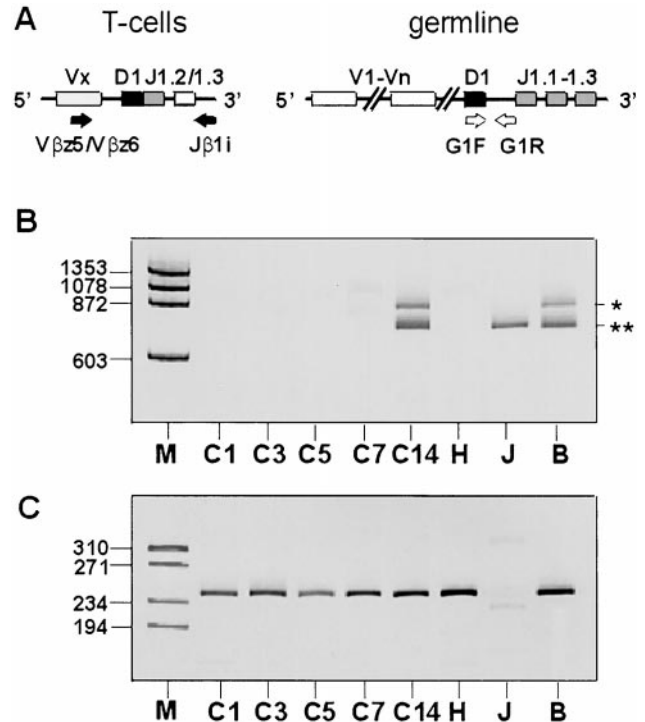


Fig. 2. Presence of T-cell DNA in plasma of cancer patients. A, position of PCR primers in the region of human T-cell receptor β chain in mature T-cell or germline configurations. B, representative examples of mature T-cell PCR. As controls we used DNA of HeLa cells, Jurkat T cells, and WBCs (Lanes H, J, and B, respectively). Lanes C1, C3, C5, C7, and C14, T-cell PCR of plasma DNA from cancer patients (see Table 1). The products of 907 (*) and 767 bp (**) represent the PCR products of two different clonal rearrangements (J1.1 and J1.2, respectively). C, germline PCR of the same samples. M, molecular weight marker in bp.

methylation status of the human *CDKN2A* gene promoter. Gene *CDKN2A* encodes a cyclin-dependent kinase inhibitor, p16^{INK4A}, with an important regulatory function in the cell cycle (25). It is known that the *CDKN2A* gene promoter is hypermethylated and thereby inactivated in a large number of diverse human cancer types (26, 27). The advantage of this approach is that unmethylated and hypermethylated DNA can be assayed side by side, allowing an estimation of the fraction of tumor-specific DNA in the samples examined. The experimental procedure is based on the deamination of cytosin, but not of 5-methylcytosin residues, by treatment of DNA with sodium bisulfite (17). Thus, an unmethylated CpG island, upstream of the *CDKN2A* gene, acquires a number of uracil residues, whereas a methylated sequence retains its 5-methylcytosines after bisulfite treatment. Consequently, different sets of primers specifically amplify unmethylated and methylated DNA. We used the primer pairs designed by Herman *et al.* (17) for the amplification of normal and hypermethylated *CDKN2A* promoter sequences. With these tools we examined a total of 25 plasma DNA samples and found evidence for hypermethylation of the *CDKN2A*-gene promoter in 11 cases (44%; Table 1), a percentage that is in agreement with published studies (26–32). In the six cases in which both plasma and tumor tissue were available, the results of the methylation-specific PCR were identical in corresponding plasma and tissue samples. Most of the positive plasma DNA samples contained DNA with both methylated and unmethylated *CDKN2A*-promoter sequences. As mentioned, the clear differentiation between unmethylated nontumor and methylated tumor DNA segments in the same plasma DNA sample allows a real-time PCR quantitation by the LightCycler technology (Fig. 3A). The results of six experiments are shown in Fig. 3B and reveal that the fraction of DNA with hypermethylated *CDKN2A*-promoter sequences varies from >90% (sample C10) to <10% (sample 23). Interestingly, samples with a high percentage of tumor-specific hypermethylated

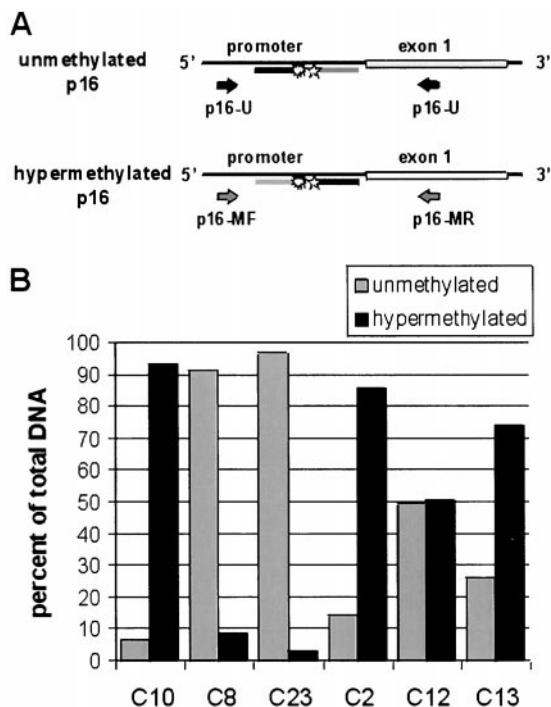


Fig. 3. Quantitation of the tumor-derived DNA in plasma by analysis of methylated and unmethylated *CDKN2A* alleles. *A*, position of sequence-specific primers and hybridization probes used in the unmethylated or the methylated LightCycler reactions. *B*, percentages of methylated (tumor DNA) and unmethylated (nontumor cell DNA) *CDKN2A* sequences in plasma of six cancer patients as analyzed by real-time quantitation. Samples C10, C8, and C23 are from advanced breast carcinoma cases (Table 1).

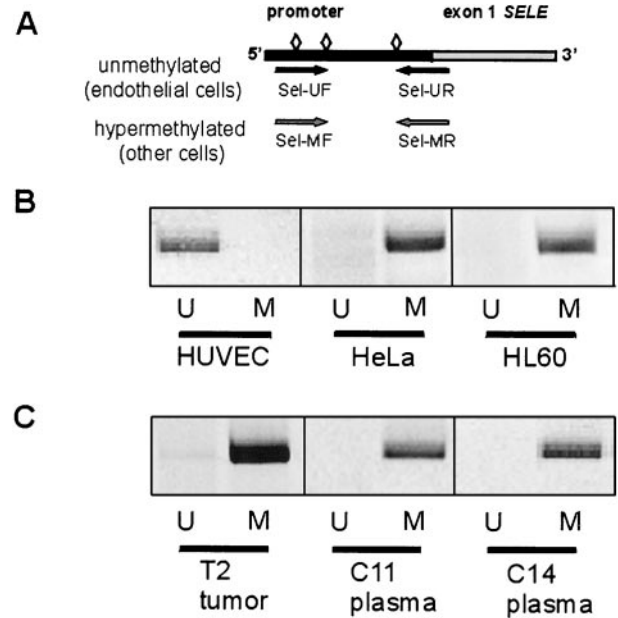


Fig. 4. Methylation-specific PCR of the promoter region of *SELE* gene. *A*, scheme for the unmethylated and methylated PCR reactions. The sequence-specific primers cover three CpG islands (\diamond). *B*, methylation-specific PCR of controls: HUVECs, HeLa cells, and HL60 cells. *C*, methylation-specific PCR of representative samples: tumor tissue of patient T2 and plasma samples of cancer patients C11 and C14. Lanes U and M, unmethylated and methylated reactions, respectively.

DNA tend to belong to a group with lower than average concentrations of circulating DNA: C10 (from a breast cancer patient with 20 ng DNA/ml plasma), C2 (angiosarcoma patient with only 19 ng DNA/ml), and C13 (melanoma patient with 20 ng DNA/ml plasma; Fig. 3B; Table 1). In contrast, sample C23 (breast cancer) has <10% tumor-specific hypermethylated DNA in a total of 360 ng DNA/ml plasma.

Where does the nontumor fraction of circulating DNA come from? A likely possibility is that it originates from degenerating normal cells in the vicinity of the expanding carcinoma tissue. This is difficult to investigate because DNA markers that distinguish defined cell types are not available. An exception may be the promoter of the endothelium-specific human gene *SELE* that has been described as unmethylated in endothelial cells but hypermethylated in other cells (33). The human gene *SELE* encodes the endothelial leukocyte adhesion molecule 1, a typical membrane component of endothelial cells (34). We confirmed the earlier observation studying DNA from human cell lines. The procedure used was methylation-specific PCR with primers corresponding to the sequences around the CpG islands upstream of the *SELE* gene (Fig. 4A). Following the reasoning explained above, we found the gene promoter unmethylated in a HUVEC line and hypermethylated in human HeLa cells and HL60 cells (Fig. 4B). Using the same PCR procedure for an investigation of surgically removed tumor tissue, we always detected a small fraction of unmethylated DNA in the presence of a much larger fraction of methylated *SELE* gene promoter DNA (Fig. 4C). This was expected, because endothelial cells constitute a small fraction of the cells in a surgical preparation. In contrast to DNA from tumor tissue, we found no evidence for unmethylated C8, C11, C14, C23, T2, T3, T6, and T7 (Fig. 4C; Table 1). We, therefore, conclude that very little, if any, DNA in the plasma DNA samples of cancer patients derives from the degeneration of endothelial cells.

Does Free DNA Originate from Apoptotic or Necrotic Cells?

We have analyzed the size distribution of purified plasma DNA from six of the cancer patients by PAGE. The DNA was visualized by

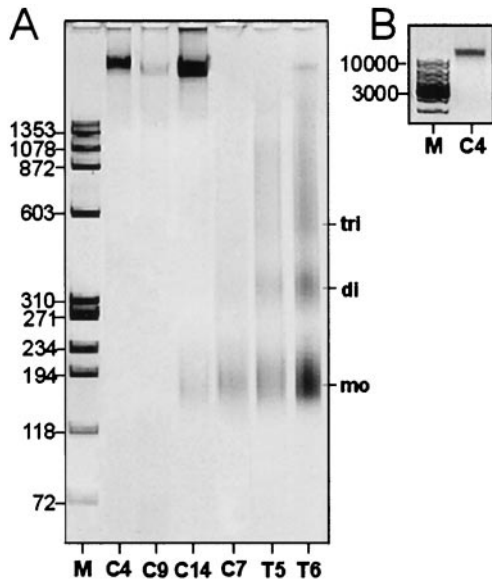


Fig. 5. A, size distribution of purified plasma DNA fragments; results of the six cancer patients. Mononucleosomal (*mo*), dinucleosomal (*di*), and trinucleosomal (*tri*) DNA fragments. B, size determination of the large-molecular-weight DNA of patient C4. M, molecular weight marker in bp.

ethidium bromide staining when sufficient amounts of DNA could be recovered. The size distribution of the DNA fragments varied from sample to sample, but most frequently, the observed size of the DNA fragments was ~ 180 bp, sometimes accompanied by DNA fragments two, three, or four times this size (Fig. 5A; patients C7, T5, and T6). We also detected high-molecular-weight DNA fragments in some samples (Fig. 5, A and B; patients C4 and C9). Plasma DNA of patient C14 consisted of both types of fragment sizes. The spectrum of multiples of 180-bp fragments is reminiscent of the oligonucleosomal DNA ladder characteristic for apoptotic cell death when cellular chromatin is degraded by a caspase-activated DNase (35). On the other hand, DNA fragments larger than $\sim 10,000$ bp could originate from cells dying via necrosis.

We, therefore, performed model studies to determine whether DNA can in principle be released from dying cells in form of soluble fragments, because this would be a necessary condition for its appearance in the circulation. In a first set of experiments, we induced apoptosis and necrosis in human Jurkat T cells. As shown in Fig. 6A, chromatin began to appear in the supernatant 3 h after induction of both necrosis or apoptosis.

It is known, however, that degenerating apoptotic and necrotic cells *in vivo* are efficiently taken up by macrophages (36), and the possibility must be considered that fragmented chromatin is so rapidly removed that it cannot appear in the circulation. To investigate this point, we used established murine models for the induction of liver cell apoptosis or necrosis by α CD95 and acetaminophen, respectively (21, 22, 37). As shown in Fig. 6B (left), we could detect a dramatic increase of DNA in plasma of mice treated with α CD95. The appearance of plasma DNA 4 h after induction of apoptosis paralleled the increase in plasma ALT activity, which reached a maximum after 8 h (2,790 IU ALT). Furthermore, the sizes of DNA fragments in the plasma corresponded to mono- and dinucleosomal DNA (Fig. 6B, right). The results of plasma DNA quantitation of mice after treatment with acetaminophen is shown in Fig. 6C (left). The increase of plasma DNA occurred with similar kinetics and was even more dramatic compared with the apoptosis model, reaching levels of >150 $\mu\text{g}/\text{ml}$ plasma 6 h after treatment of mice. At this time point, the extent of liver injury was maximal, as expressed by a plasma ALT level of 2200

IU ALT. In contrast to the mono- and dinucleosomal DNA fragments appearing in the plasma of α CD95-treated mice, we here found a time-dependent increase of DNA fragments $>10,000$ bp, as expected for necrotic cell death (Fig. 6C, right). These results support the idea that the DNA in the circulation of cancer patients could originate from both apoptotic and necrotic cells in cancer tissue and that a discrimination of DNA originating from either type of cell death is possible by the determination of DNA size distribution.

DISCUSSION

In this study, we investigated the plasma of cancer patients and detected, in agreement with previous research, concentrations of DNA that are on the average much higher than the DNA levels in the plasma of healthy controls (1–4). The range of DNA levels in the circulation of cancer patients varies widely, from levels like those in some of the controls (10–20 ng/ml) to levels that exceed values of 1000 ng DNA/ml plasma. The levels of plasma DNA show no obvious corre-

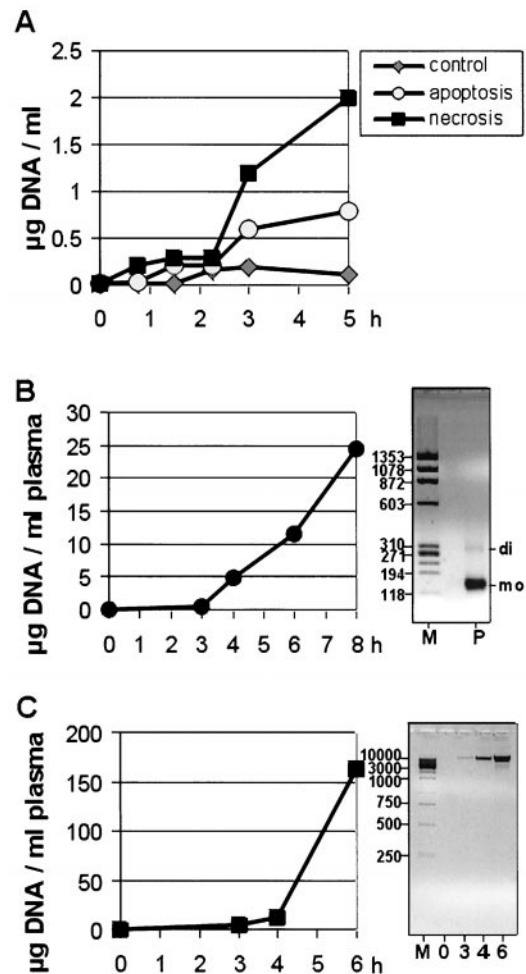


Fig. 6. Extracellular DNA after induction of cell death *in vitro* and *in vivo*. A, supernatant DNA quantities of apoptotic or necrotic Jurkat T cells. Apoptosis was induced by staurosporine; necrosis was induced by staurosporine plus oligomycin (14). DNA in supernatants was quantitated by competitive PCR at the time points indicated. B, apoptosis *in vivo*. Mice were treated with anti-CD95 antibody (17). Quantities of plasma DNA analyzed by real-time PCR quantitation (averages from three animals per time point). Right: Lane M, DNA fragments of known sizes (in bp); Lane P, size distribution of plasma DNA fragments at 8 h after injection of anti-CD95 antibody (*mo* and *di*, mononucleosomal and dinucleosomal DNA fragments, respectively). C, necrosis *in vivo*. Mice were treated with acetaminophen (17), and plasma DNA was analyzed by real-time PCR quantitation at the time points indicated (averages from three animals per time point). Right: Lane M, DNA marker (in bp); Lanes 0, 3, 4, and 6, plasma DNA at 0, 3, 4, and 6 h after injection of acetaminophen, respectively.

lation to the type of cancer or to the clinical status. The variations in the amount of total DNA in patients with a similar clinical status were also found in studies of other laboratories with different tumor types (1, 2, 13) and could possibly be influenced by differences in the catabolism of plasma DNA in different patients. The difference between the levels of plasma DNA in healthy individuals and cancer patients is more extreme than the results of other studies (1–4, 7). Our explanation for this finding is that our quantitation assay is based on PCR amplification, which could be influenced by the fragmentation grade of the DNA. We, therefore, cannot exclude that some of the plasma DNA fragments were not amplified in our assay, so that the amounts of DNA could probably be higher.

Plasma DNA can originate from both tumor and nontumor cells. We found that neither T cells nor endothelial cells seem to contribute to plasma levels of DNA, with the exception of two cancer patients who had T-cell DNA in their plasma samples. We conclude that the nontumor DNA in human plasma originates from other cells surrounding the tumor tissue that are degenerated by the growing tumor (38). To determine the ratio of tumor and nontumor DNA, we measured the hypermethylation of the *CDKN2A* gene promoter, an epigenetic DNA modification that is characteristic for the DNA in many cancer cell types (27). We could detect hypermethylation in >40% of the samples studied, a value that agrees well with reports in the literature (26–32). Quantities of hypermethylated tumor-derived DNA and unmethylated DNA from nontumor cells were determined by parallel quantitative methylation-specific PCR assays, showing that some samples of plasma DNA consisted of a high proportion of tumor cell DNA, whereas others consisted predominantly of DNA from nontumor cells. A case in point are samples C10, C8, and C23, which were all taken from advanced breast cancer patients with metastases but show different percentages of tumor-derived DNA. We estimate, however, that the absolute amounts of tumor-derived DNA are similar, ranging from 7 to 18 ng/ml plasma. In fact, in the six cases that we have studied, the concentration of tumor-derived DNA is generally low: <20 ng/ml, with a range of 5 to 18 ng/ml. Studies from other laboratories have investigated microsatellite loss of heterozygosity in plasma DNA and concluded that in at least some cancer patients nearly the whole plasma DNA is derived from the tumor cells (7, 9). This contrasts with other studies that detected wild-type DNA in the plasma of nearly all of the cancer patients (13). In fact, many studies had to use very sensitive methods such as mutant allele-specific PCR (MASA) or RFLP PCR to detect mutations in plasma DNA samples of cancer patients (6, 39). Here we describe that the fraction of tumor-derived DNA appears to be higher when the total level of plasma DNA is low. This could lead to the conclusion that the fraction of tumor DNA in plasma may be correlated with the state of tumor progression: small tumors shed small amounts of DNA; larger tumors that infiltrate the surrounding normal tissue shed more DNA including that from adjacent nontumor cells. However, the data presented here do not seem to support this possibility, because similar clinical situations are associated with widely differing amounts of circulating tumor as well as nontumor DNA levels. Although a larger and more systematic study is necessary to decide this issue, it appears that although cancer does usually lead to an increase of plasma DNA, its amount and composition varies between patients. This may not be surprising given the multitude of reactions that are expected to occur from the time when DNA is released from degenerating cells until its eventual appearance in the circulation.

Our studies with cell cultures and animal models show that soluble DNA in the form of chromatin fragments are released from apoptotic and necrotic cells and can eventually appear in the blood stream. We, therefore, assume that the source of DNA in the blood plasma of cancer patients are cells that disintegrate by apoptosis and/or necrosis

in expanding tumor tissue. It is possible to distinguish between the two models of cell death: apoptosis produces DNA fragments of 180 bp (and multiples of this), whereas necrosis results in much larger fragments. Both types of fragment sizes are found in the blood stream of cancer patients. In some of the analyzed cancer patients we found no evidence for apoptotic DNA. In fact, necrosis of tumor cells has been postulated to be the origin of plasma DNA in cancer patients in a number of earlier studies (2–4, 13), including one study that showed an increase of plasma DNA after radiation therapy (40). However, we have shown here that apoptosis of cancer cells is at least as likely to lead to increased plasma DNA levels.

As a conclusion, we envisage the following scenario. As the size of a tumor increases, vascularization becomes a problem, causing hypoxia in regions remote from blood vessels. Hypoxia induces *p53*-dependent or *p53*-independent apoptosis of tumor cells and of nontumor cells in the infiltrated tissues (41), just as cells may die by necrosis. Dead cells are normally removed by phagocytes (36, 42), but this process may not be efficient in and around tumors, or some fraction of the released soluble chromatin fragments may escape clearance by phagocytes and find its way into the blood stream. Clearly, several complex processes determine the fate of the DNA released from degenerating cells in tumor tissue, and it can be expected that the efficiencies of these processes depend on many variables. This may explain the large variation in the amounts and composition of DNA found in the circulation of cancer patients.

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DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells

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