

# An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells

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## Abstract

Non-invasive screening that utilizes cell-free DNA (cfDNA) offers remarkable potential as a method for the early detection of genetic disorders and a wide variety of cancers. Unfortunately, one of the most prominent elements delaying the translation of cfDNA analyses to clinical practice is the lack of knowledge regarding its origin and composition. The elucidation of the origin of cfDNA is complicated by the apparently arbitrary variability of quantitative and qualitative characteristics of cfDNA in the blood of healthy as well as diseased individuals. These factors may contribute to false positive/negative results when applied to clinical pathology. Although many have acknowledged that this is a major problem, few have addressed it. We believe that many of the current difficulties encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. The results obtained in this study indicate that the release of cfDNA from 143B cells is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily the result of actively released DNA, perhaps in association with a protein complex. Moreover, this study demonstrates the potential of *in vitro* cell culture models to obtain useful information about the phenomenon of cfDNA.

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**Keywords**

Cell-free DNA (cfDNA) • Apoptosis • Necrosis • Osteosarcoma • Flow cytometry

**Introduction**

Understanding the processes involved in the generation of cell-free DNA (cfDNA) is critical for deducing its role in biology and pathology, while advancing the translation of analyses to clinical practice. However, the origin of cfDNA still remains elusive despite the seemingly universality of it in bio-fluids. Several sources of cfDNA have been excluded including, exogenous DNA (bacterial, viral and parasitic), lysis of cells on the interface between a tumour and circulation (Sorenson 1997) and the destruction of tumour micrometastases and circulating cancer cells (Bevilacqua et al. 1998). Currently, the only remaining conceivable sources that may account for the occurrence of cfDNA are apoptosis, necrosis, or active cellular secretion (Stroun et al. 2001).

Although most evidence suggests that the release of cfDNA is mainly a consequence of apoptosis, many studies have indicated that a significant fraction of cfDNA is derived from active cellular secretions (van der Vaart and Pretorius 2008), where newly synthesized DNA in association with a lipid-protein complex is released in a homeostatic manner (Anker et al. 1975; Borenstein and Ephrati-Elizur 1969; Stroun and Anker 1972; Stroun et al. 1977, 1978). The aim of this work was to examine the release and composition of cfDNA from cultured human osteosarcoma cells (143B). The release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments evaluated at each of these intervals. Additionally, apoptotic, necrotic and the cell cycle profiles were also investigated using flow cytometry.

**Materials and Methods****Cell Culturing, Extraction and Quantification of Cellular Protein and Cell-Free DNA**

The human osteosarcoma cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL- 8303TM). Cells were cultured as prescribed by ATCC. Total cellular protein was isolated and quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies). cfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions and quantified using real-time quantitative PCR assay for the  $\beta$ -globin gene.

**Fragment Size Evaluation of Cell-Free DNA**

The size of cfDNA extracted at the different time intervals were analysed by capillary electrophoresis using the High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. The assay was performed according to the instructions provided by the manufacturer.

**Flow Cytometric Assays**

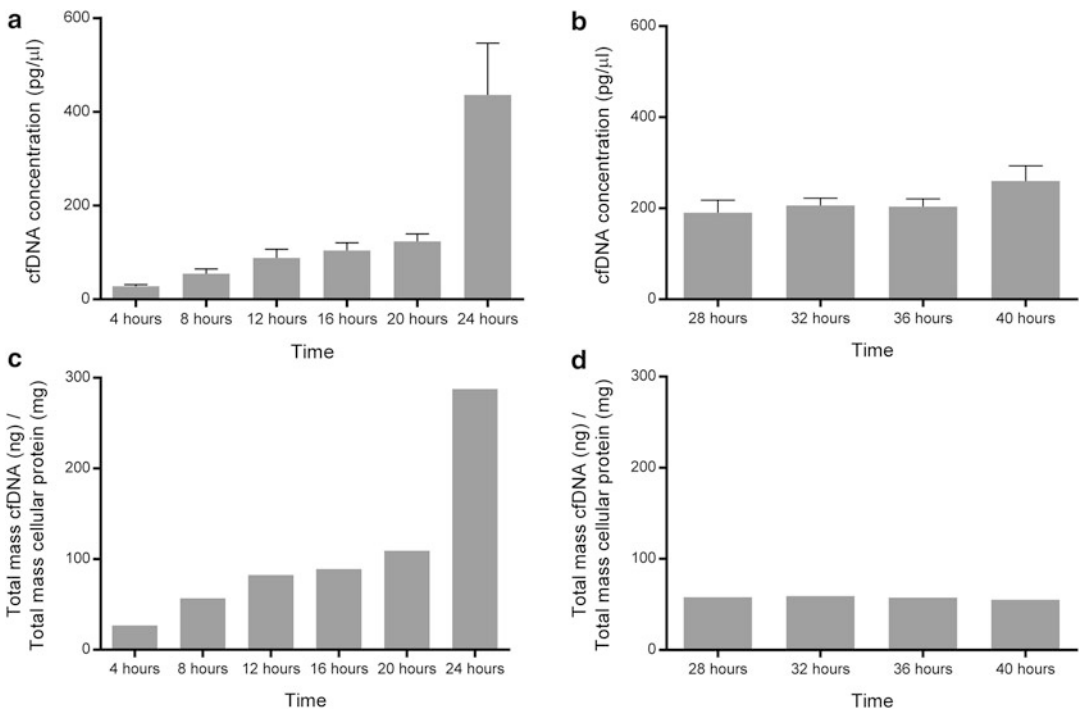
The BD Annexin V FITC assay (BD Biosciences) was utilized to determine the apoptotic/necrotic profile of 143B cells. An

APO-BrdU<sup>TM</sup> TUNEL assay kit (Molecular Probes, Invitrogen) was used for the detection of DNA fragments as recommended by the manufacturer. For cell cycle analysis the Click-iT<sup>®</sup> Edu Alexa Fluor<sup>®</sup> 488 Cell Proliferation Kit (Molecular Probes, Invitrogen) was utilized according to the instructions of the manufacturer. All flow cytometric analyses were done on a FACSVerser<sup>TM</sup> bench top flow cytometer. Events were acquired on BD FACSuite<sup>TM</sup> software (Becton & Dickson, Mountain view, CA, USA).

## Results and Discussion

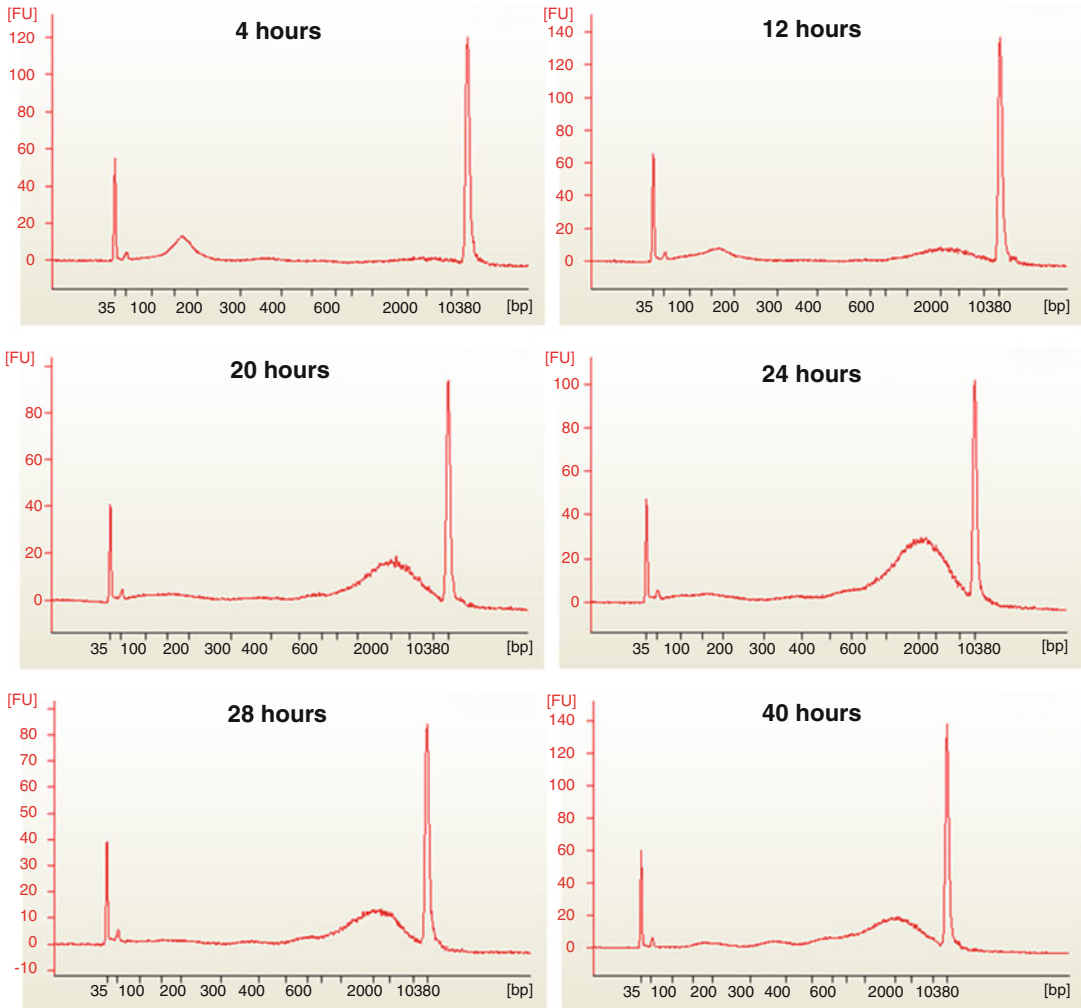
In this study, the release of cfDNA from cultured cells was evaluated in order to gauge its potential use for elucidating the nature of cfDNA. Release of cfDNA from 143B cultured cells was characterized over time (Fig. 4.1a, b).

After growth medium renewal, the amount of cfDNA increased incrementally, notably peaked after 24 h, and plateaued at a much lower level thereafter. Since the amount of cells increase over time, this is not surprising. However, when the values were normalized in terms of total cellular protein the tendency did not change, suggesting that more cfDNA is released per cell in a time dependent manner (Fig. 4.1d, c). This suggested that the cfDNA is neither from apoptotic nor necrotic origin but originates from active release. To examine the size of cfDNA isolated at the different time points, samples were subject to capillary electrophoresis (Fig. 4.2). After 4 h of incubation a prominent peak at 166 bp, however, this peak dissipated incrementally and disappeared after 24 h when a new peak of approximately 2000 bp dominated the scene. After 40 h of incubation, the cfDNA resembles multiples of nucleosomal repeats, with small populations forming at approximately 160, 340 and 540 bp,



**Fig. 4.1** Time-course characteristics of cfDNA released from 143 B cells. (a) Bar graph showing the amount of cfDNA released by 143 B cells after 4–24 h of incubation following medium renewal. (b) Bar graph showing the

amount of cfDNA released by 143B cells after 28–40 h of incubation following medium renewal. (c) & (d) Represents the amount of cfDNA released at each time-point normalized in terms of total cellular protein



**Fig. 4.2** Capillary electropherograms showing the size of cfDNA isolated after incubation at various times following medium renewal. In each electropherogram two major peaks can be seen, one at 35 bp and one at approximately 10 000 bp. These peaks correspond to the two size mark-

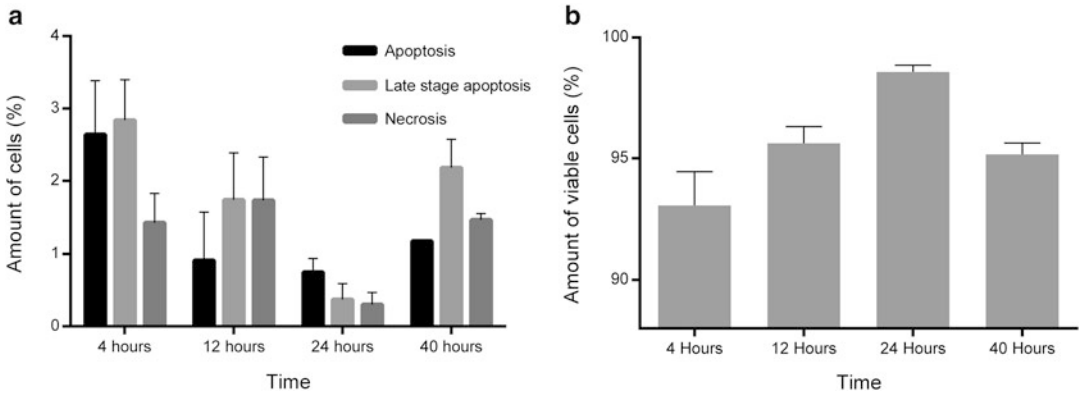
ers. The relative fluorescence of these markers is then used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA

suggesting an apoptotic origin. These observations demonstrate a clear correlation between an increase in the release of cfDNA and the occurrence of higher molecular weight DNA. As far as we know, this distinct size of ~2000 bp has not yet been reported. This is noteworthy, because its size suggests that it is neither from apoptotic nor necrotic origin.

To verify the observations made by electrophoretic analysis, and to help elucidate the origin of the cfDNA present at the different time points,

the cells at each of the times correlating with the time-course study was analyzed for apoptosis, necrosis and cell cycle phase using flow cytometric assays. The FITC Annexin V assay revealed that a fraction of cells are apoptotic after 4 h, whereas 24 h showed almost no apoptosis or necrosis (Fig. 4.3).

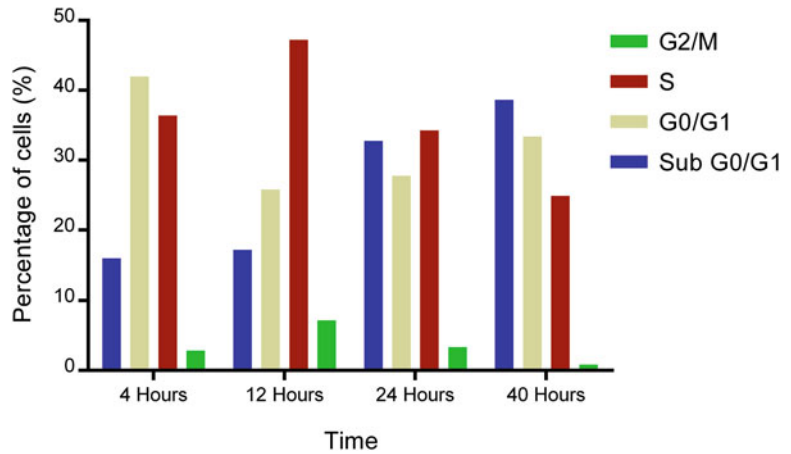
Excluding the 4 h time point, these results were corroborated by the TUNEL assay, which is based on the detection of fragmented DNA (results not shown). The assay used to measure



**Fig. 4.3** Summary of data obtained by the FITC Annexin V flow cytometric assays. (a) illustrates the percentage of apoptotic, necrotic, and late-stage apoptotic 143 B cells at

the different time-points. (b) illustrates the percentage of viable 143 B cells at each time-point. Error bars indicate standard deviation

**Fig. 4.4** Bar graph representing the cell cycle distribution of 143 B cells at different points in time



cell proliferation revealed that, after 24 h of growth following medium renewal, there is a decline of cells in the S phase and significant increase of cells in the sub G0/G1 phase (Fig. 4.4). This indicates that the abrupt increase of cfDNA after 24 h of incubation is not associated with the process of DNA replication.

To determine whether the cfDNA released from 143 B cells after 24 h of incubation could be similar to virtosomes, growth medium was treated with denaturing agents prior to cfDNA extraction, and were compared to untreated samples (results not shown). In all cases, the concentration of cfDNA was increased considerably by the addition of denaturing agents. These results suggest that cfDNA may be asso-

ciated with proteins but whether these proteins are simply nucleosomes, lipid-protein complexes (virtosomes) or extracellular vesicles remains unclear, and requires further examination.

## Conclusion

Most *in vivo* studies report that the occurrence of cfDNA is associated with apoptosis or necrosis. However, the results obtained by this study suggest that the release of cfDNA from cultured 143B cells after 24 h of incubation is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily a result of actively

released DNA, perhaps in association with a protein complex. These results also demonstrate the potential of *in vitro* cfDNA analysis to aid in the elucidation of the nature of cfDNA.

**Acknowledgement** AB and JA were supported by post-graduate scholarships from the National Research Foundation (NRF) South Africa. The financial assistance of the NRF is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not to be attributed to the NRF. We would also like to thank the North-West University, Potchefstroom, South Africa for additional financial support.

**Conflict of Interest** The authors wish to declare no conflict of interest.

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## References

- Anker P, Stroun M, Maurice PA (1975) Spontaneous release of DNA by human blood lymphocytes as shown in an *in vitro* system. *Cancer Res* 35(9):2375–2382
- Bevilacqua RA, Nunes DN, Stroun M et al (1998) The use of genetic instability as a clinical tool for cancer diagnosis. *Semin Cancer Biol* 8:447–453
- Borenstein S, Ephrati-Elizur E (1969) Spontaneous release of DNA in sequential genetic order by *Bacillus subtilis*. *J Mol Biol* 45(1):137–152
- Sorenson G (1997, September) Communication at the XXVth anniversary meeting of the international society for oncodevelopmental biology and medicine. Montreux, Switzerland.
- Stroun M, Anker P (1972) Nucleic acids spontaneously released by living frog auricles. *Biochem J* 128(3):100P
- Stroun M, Anker P, Gahan PB et al (1977) Spontaneous release of newly synthesized DNA from frog auricles. *Arch Des Sci* 30(2):229–241
- Stroun M, Anker P, Beljanski M et al (1978) Presence of RNA in the nucleoprotein complex spontaneously released by human lymphocytes and frog auricles in culture. *Cancer Res* 38(10):3546–3554
- Stroun M, Lyautey J, Lederrey C et al (2001) About the possible origin and mechanism of circulating DNA: apoptosis and active DNA release. *Clin Chim Acta* 313(1):139–142
- van der Vaart M, Pretorius PJ (2008) Characterization of circulating DNA in healthy human plasma. *Clin Chim Acta* 395(1):186