The Biology of CNAPS

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Abstract Although nucleic acids have been known to circulate in the blood since 1948 their biology has been studied only since the 1960s. This chapter contains discussion of (a) the presence of DNA and RNA circulating in human plasma and serum from both healthy individuals and patients, (b) the amounts of DNA/RNA present together with the variables affecting these amounts, (c) possible sources of the DNA/RNA in blood and (d) the ability of the circulating nucleic acids to enter other cells and to modify the biology of the recipient cells. The relationship of the DNA from cancer patients is considered with respect to the formation of metastases.

Keywords Circulating DNA/RNA • Sources • Entry into/exit from cells • Cell modification • Metastases

1 Introduction

The background concerning the presence of cfDNA/cfRNA in blood and other body fluids has been considered in chapter "Brief History and the Present and Future Status of CNAPS". Developments in analyzing these cfNAs have permitted the study of various applications of their use in diagnosis, prognosis and monitoring of treatment of clinical disorders. Since the first identification of cfDNA and cfRNA, there has been an increase in the general identification of the members of the RNA family and in a broadening of their analysis in CNAPS. This chapter will consider the biology of cfNAs in plasma and serum as well as in urine, saliva, cerebrospinal fluid and amniotic fluid. The possible cellular origins of cfDNA/cfRNA found in

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blood, the mechanisms of release into the blood and their uptake into other cells as well as their possible biological effects in the host organism will also be considered.

2 cfNucleic Acids and Nuclease Content

Both DNA (1.8–15 ng mL^{-1}) and RNA (2.5 ng mL^{-1}) are found in plasma and serum from healthy donors [1 –3]. These levels rise in patients with various cancers, trauma, myocardial infarction and stroke with values of 3,000 μg DNA mL^{-1} and above being recorded on occasions [4]. In consequence, the amount of cfDNA and cfRNA present in plasma and serum will depend upon the health status of the individual and will also be influenced by the amount of nucleases present in blood.

The average blood plasma concentration of DNAase 1 forms 90 % of total blood DNase i.e. 41 ± 30 ng mL^{-1} for healthy men and 21 ± 21 ng mL^{-1} for healthy women yielding an activity of 0.307 ± 0.249 U mL^{-1} for men and 0.405 ± 0.509 U mL^{-1} for women. In contrast, the values for diseased individuals rise with e.g. gastrointestinal cancer patients having about 350 ng mL^{-1} [5]. The average serum RNAase value for 54 normal individuals was 104 units mL^{-1} while for those suffering from pancreatitis was 120 units ml^{-1} and pancreatic cancer was 383 units mL^{-1} [6]. Based on such measurements, it can be postulated that the relatively low levels of circulating cfDNA in healthy individuals could occur, partially, due to peripheral blood DNAase activity. However, the DNA of cancer patients could be resistant to DNAase as demonstrated by using bacterial DNAase [7]. This could be possibly due to either accessory (lipo)-protein and/or low DNAase levels. Equally, high RNA levels may also be due to RNA resistance to RNAase digestion especially when high RNAase and RNA levels co-exist. The RNA may either be protected by a glycolipid resulting from its apoptotic origin [8] or the DNA and RNA fractions are associated with the exosomes and virtosomes from living cells so protecting the cfNAs from digestion by RNAase/DNAase activity ([1, 9–13]; section "Exosomes"/"Virtosomes").

3 Nucleic Acid Sources

The presence of DNA in the blood raised the question as to its origin. The most obvious suggestion was that it must be derived from dead and dying cells. However, whilst this was a likely source, experiments by Stroun and Anker demonstrated that healthy, living cells also could release DNA and RNA [1, 11, 14–16]. Subsequent analyses have indicated that there are at least twelve possible sources of blood cfNAs (Table 1).

Table 1 Possible sources of cfDNA and cfRNA circulating in plasma and serum

DNA and RNAs[a]
1. Leukocyte breakdown
2. Bacteria breakdown
3. Viruses
4. Mitochondrial DNA
5. Cell and tissue necrosis
6. Cell apoptosis
7. Cellular release of exosomes
8. Cellular release of RNAs
9. Cellular release of virtosomes
10. Parasite nucleic acids
DNA
1. Cellular release of transposons and retrotransposons
2. Leukocyte surface DNA

[a]Including the many forms of RNA (see Sect. 3.2) though to date, the cfNA focus has been primarily on mRNAs and miRNAs

3.1 cfDNA

Leukocytes

A minimal amount of DNA is released on the death of leukocytes and is unlikely to account for the total amounts of cfDNA found in normal blood i.e. 1.8–36 ng mL^{-1} [1, 5, 17] and certainly not the levels found during clinical disorders; larger amounts have been reported for both normal individuals and those suffering from e.g. cancer [4].

However, DNA can be found on the surface of leukocytes through either trapping DNA on the outer surface prior to its internalization and destruction or secretion of a DNA network onto the surface [18–21]. Neutrophils can release sticky webs of chromatin (DNA + histones) during infection to trap invading microbes. These neutrophil extracellular traps have granules containing lytic enzymes and antimicrobial peptides exploited for the rapid killing of invading pathogens [22–24].

In addition, lipopolysaccharide from Gram-negative bacteria activates either interleukin-5 (IL-5)- or interferon-gamma primed eosinophils leading to a release of mitDNA independently of eosinophil death. The mitDNA is rapidly (<1 s) released involving a catapult-like movement. mitDNA, together with protein, forms the extracellular network that is believed to bind and kill bacteria in vitro and in vivo under inflammatory conditions [25]. However, Menegazzi et al. [26] have challenged this explanation suggesting that the DNA network was released only by dying neutrophils. This is based upon experiments in which live bacteria were released from the DNA network on treatment with DNAase.

Importantly, cell surface DNA forms the basis of a number of early diagnostic and monitoring analyses (e.g. [27]).

Bacteria and Viruses

The levels of DNA seen are unlikely to be due to the presence of bacteria found normally due to their low numbers. Some viral DNAs have been recorded in peripheral blood e.g. Epstein Barr virus (EBV) in patients with nasopharyngeal carcinoma [28] and human papilloma viral carcinoma in about 50 % of cervical carcinoma patients and hepatitis patients [21, 29]. Human herpes virus-6 (HHV-6) DNA has also been found in both plasma and serum. HHV-6 DNA in plasma was readily attacked by DNAase and so is considered to be unencapsulated. HHV-7 was also identified in plasma, but only in the P1 sub-fraction whereas HHV-6 appeared in P1, P2 and S sub-fractions, especially in P1 [30].

That bacteria and viruses are likely to form only a very small part of cfDNA/cfRNA was shown in a study by Beck et al. [31] on blood from 51 apparently healthy individuals when they obtained 4.5×10^5 DNA sequences (7.5×107 nucleotides). Of these, 87 % were attributable to known human database sequences and only 3 % were found to be xenogeneic.

Necrosis

Necrosis has also been considered as a potential source of blood cfDNA. The cfDNA derived by necrosis forms non-specifically and incompletely digested pieces in excess of 10,000 bp and, unlike apoptotic DNA fragments, forms smears when electrophoretically run on gels [32–34]. The cfDNA fragments released from necrotic cells and present in blood plasma samples have been shown, via electrophoresis and sucrose-gradient sedimentation, to contain high molecular weight DNA fragments ranging from 21 kb [35] to 80 kb in length [36]. Hence, necrosis does not appear to be a major contributor to cfDNA, the electrophoretic separation pattern showing primarily an apoptotic ladder pattern rather than the smear pattern.

Apoptosis

This would appear to present a major contributor to cfDNA. cfDNA in blood is double-stranded [1, 37] and forms a ladder pattern when separated by agarose gel electrophoresis [32, 38, 39] with fragment sizes ranging from 60 to 1,000 bp. The fragment ends are capped showing them to be present in the form of nucleosomes or apoptotic fragments. This has led to the cfDNA being considered as the apoptotic product from e.g. tumors. The typical electrophoretic ladder pattern of DNA from apoptotic cells is initially of 50–300 kb fragments that mainly fragments into multiples of nucleosomal fragments (180–200 bp). Thus, the cfDNA fragments may have their origin via apoptosis [36, 38, 40]. Apoptotic fragments are expected to be phagocytozed by macrophages and dendritic cells at the final stage [41] and so

should not be released into the blood. It has been suggested that if the release is from solid tumors there could be a breakdown in the phagocytic process [40, 42, 43].

Further evidence for the apoptotic origin of DNA is through the identification of mitDNA in CNAPS [43]. Both particle-associated and mitcfDNA are present in plasma, their respective concentrations being modified depending on the preparation of plasma from whole blood [44]. Thus, mitDNA increases were found in trauma patients [45, 46], with median plasma mitDNA concentrations having double the number of copies mL^{-1} in the severely injured subgroup compared with the minor/moderate subgroup. A 2.6-fold increase in mitDNA was found in patients dying from prostate cancer as opposed to those who survived [47]. The median mitDNA copies 100 μL^{-1} plasma for prostate cancer patients were 49,193 compared to 19,037 for benign controls.

Exosomes

These membrane-bound structures, 30–90 nm in diameter, are secreted by most cell types and may play a role in intercellular signaling. Although originally described in 1983, interest in exosomes has increased recently due to them containing both mRNA and miRNA ([48] see section "Exosomes"). DNA has also been found to be present in exosomes of both man and mouse [49–51]. DNA-containing exosomes have been linked to the initiation of both glioma and colorectal cancer under experimental conditions [49, 50]. Nevertheless, exosomes appear to be low-level contributors of DNA/RNA to cfNAs.

Virtosomes

A further contributor to CNAPS would appear to be newly synthesized DNA that has been shown to be spontaneously released, in a regulated manner, from both stimulated and non-stimulated human [1, 52, 53] and rat lymphocytes [9, 10] in vitro. This DNA is complexed with newly synthesized lipoprotein [9, 10, 54] and newly synthesized RNA [9, 10] i.e. all of the components of this complex are newly synthesized. This complex has been termed a virtosome.

The release of newly synthesized DNA from living, but not dead or damaged cells, has been shown to be of general occurrence in vitro (Table 2) as well as being released in vivo from whole chick embryos [59].

Transposons and Retrotransposons

These mobile genetic elements, or transposable elements, form a substantial part of the nuclear "c" DNA value.

Table 2 Release of newly synthesized DNA from prokaryote and eukaryote cells and tissues

Cell/tissue	References
Bacteria	Ottolenghi and Hotchkiss [55, 56] and Borenstein and Ephrati- Elizur [57]
Human stimulated and non-stimulated lymphocytes	Anker et al. [1]
Rat stimulated and non-stimulated lymphocytes	Olsen and Harris [52] and Adams and Gahan [9, 10] Adams et al. [58]
Chick embryo fibroblasts	Adams and MacIntosh [54]
Frog heart auricle pairs	Stroun and Anker [16] and Stroun et al. [11]
Frog brain	Anker and Stroun [14]

Retrotransposons copy themselves via RNA, whilst the transposons copy themselves without the intervention of RNA. The Alu repeat sequence of approximately 300 bases, being found 300,000–1,000,000 times in the human genome, is the commonest form of human transposons. ALU repeat DNA fractions have been reported to be present in CNAPS [60, 61] together with the retrotransposon LINE 1 [62]. ALU and LINE1 are distributed throughout the genome being less methylated in cancer cells as opposed to normal cells [63].

Mitochondrial DNA Release

This was found in CNAPS from trauma patients [45, 46]. Bound and mitcfDNA have both been found in plasma, the concentrations of each possibly being affected by the mode of preparation of plasma from whole blood [44]. mitcfDNA may be also derived by apoptosis [43] (see also Sect. 3.1).

Parasite DNA Release

This is a little studied area, but the possibility of parasite nucleic acids being present as cfDNA have been discussed by Gahan [64] in considering aspects of HGT.

3.2 RNA

In a similar fashion to DNA, RNA can be released from any one of ten possible sources listed in Table 1. There has been an explosion in the identification of a variety of RNAs and their various roles in cells including mRNA, tRNA, rRNA, snRNA, snoRNA, dsRNA, RNAi, siRNA, miRNA, piRNA, circRNA, ceRNA and lncRNA (see section "Exosomes"). This is reflected in the identification by deep sequencing of many of these RNAs in exosomes [65] although much of the current work on cfRNA has been essentially limited to cfmRNA and cfmiRNA.

Messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) are all involved in transferring information from chromosomal DNA to the ribosomes where amino acids are transported for the construction of protein [66]. Involved in these processes are small nucleolar RNAs (snoRNA), a class of small RNA molecules that mainly guide chemical modifications of other RNAs including rRNAs, tRNAs and small nuclear RNAs (snRNA). The latter are a class of small RNA molecules found within the eukaryotic nucleus and having an average length of ca 150 nucleotides. Their primary function concerns the processing of pre-mRNA (hnRNA) in the nucleus.

There are a variety of small non-coding RNAs involved in cfNAs including miRNA that is important in a number of studies discussed throughout this book. It involves a single-stranded RNA of 20–25 nucleotides functioning in transcriptional and post-transcriptional regulation of gene expression through binding to the 3′-untranslated region of the target mRNAs. The human genome has over 1,000 miRNAs that function via base-pairing with complementary sequences within mRNA molecules. Their action usually results in gene silencing via either translational repression or targeted mRNA degradation.

Other small non-coding RNAs include:

(i) Long double-stranded RNA (dsRNA) is cleaved into short 21–24 nucleotide double-stranded RNAs (siRNA). Each siRNA unwinds to form two single-stranded RNAs (ssRNAs), the passenger strand and the guide strand. The former is degraded while the latter is incorporated into the RNA-induced silencing complex (RISC). A well-studied example of its function involves post-transcriptional gene silencing when the guide strand base pairs with a complementary sequence of a mRNA to induce cleavage by Argonaute, the catalytic component of the RISC complex.

(ii) Similar to the siRNAs are the piRNAs, Piwi-interacting RNAs, found in gonads, are the largest class of small non-coding RNA molecules. They are distinguished from miRNA by being of 26–31 nucleotides rather than 21–24 nucleotides, lacking sequence conservation and increased complexity. The piRNA complexes, formed by interacting piRNA with piwi proteins, have been linked to silencing of both epigenetic and post-transcriptional genes of retrotransposons in germ line cells.

(iii) Long non-coding RNAs (lncRNA) are non-protein coding transcripts longer than 200 nucleotides being much longer than the above-mentioned RNAs. There are at least four-times more lncRNA than coding RNA sequences. Their roles have not been well analyzed though it has been shown that there is involvement with the physiological aspects of cell-type determination and tissue homeostasis.

(iv) Circular RNAs (circRNA) concern a class of circular RNA molecules that may play a regulatory role in miRNA processes. Initial studies have shown that they may act as “sponges” for miRNAs since e.g. circRNA (ciRS-7) acts as a miR-7 super-sponge containing ~70 target sites from the same miR-7 at the same transcript.

(v) Salmena et al. [67] propose the presence of ceRNA – competing endogenous RNA and hypothesize that "in addition to the conventional microRNA/RNA function, a reversed RNA/microRNA logic exists in which bona fide coding and noncoding RNA targets can crosstalk through their ability to compete for microRNA binding. On the basis of this hypothesis, MREs can be viewed as the letters of an "RNA language" by which transcripts can actively communicate to each other to regulate their respective expression levels. We hypothesize that RNAs that share multiple MREs will crosstalk effectively. Importantly, we predict that this "RNA language" can be used to functionalize the entire mRNA dimension through the identification of crosstalking ceRNAs, as well as ceRNA networks." ceRNAs may be involved in cfNAs since ceRNAs for the tumor suppressor gene *PTEN* have been identified for prostate cancer [68], glioma [69] and melanoma [70].

Leukocytes

Leukocyte breakdown could release a range of RNAs into the blood so possibly accounting for the RNA amount already determined [2].

Bacteria and Viruses

The presence viral RNA has been demonstrated for hepatitis C RNA in the plasma and serum of European and African patients using an RT-qPCR and the isothermal NASBA nucleic acid amplification system encompassing a gel-based detection assay. This extraction method has allowed the detection of hepatitis C RNA equally in both serum and plasma using either heparin or EDTA [71]. Majde et al. [72] have also shown the release of dsRNA into the extra-cellular medium from influenza virus-infected MDCK epithelial cells. Little information is available of the levels of RNA released from bacteria into the blood stream.

Necrosis and Apoptosis

A spectrum of mRNAs has been identified in plasma that are presumed to have been released by either apoptosis or necrosis including those representing (i) genes overexpressed in a range of different tumors [73, 74], (ii) fetal genes in the blood plasma of pregnant women [75], (iii) genes of patients with diabetic retinopathy [76] as well as (iv) housekeeping genes detected in the plasma of healthy persons [77].

RT-qPCR detectable fragments of 18S rRNA were also found in the extracellular RNA pool circulating in blood plasma of healthy subjects and cancer patients [78]. Ribosomal 28S rRNA fragments secreted by primary and cultivated human cells into the culture medium have been demonstrated [79].

Exosomes

Early studies indicated that exosomes contained RNAse that was thought to destroy unwanted RNA [66]. In fact, exosomes contain many domains with RNAase activity. Hence, if such exosomes were to be released into the blood stream, they could contribute to the RNAse levels.

More recently, it has transpired that exosomes may act as vehicles for the transfer of a variety of RNAs and so as an inter-cellular signaling system. The RNAs include mRNAs, miRNAs, non-coding RNAs, retrotransposon elements, genomic DNA and cDNA derived from oncogenic sequences [80–82]. Exosomes from both mouse and human mast cells have been shown to contain cfRNA as have primary, bone marrow-derived, mouse mast cells [82]. The presence of cfRNA in exosomes has also been reported for those released from tracheobronchial ciliated epithelial cells [83]. Importantly, exosomes released into the blood from glioblastoma patients were shown to contain mRNA mutant/variants and miRNA characteristic of gliomas. In particular, tumour-specific EGFRvIII, containing a mutation specific for glioblastoma, was detected in the serum micro-vesicles of seven out of 25 glioblastoma patients. The glioblastoma-derived vesicles were shown to stimulate proliferation of a human glioblastoma cell line [81]. These workers also showed mRNA and miRNA to be taken up by normal host cells including brain and microvascular endothelial cells.

More recently, Huang et al. [65] characterized human plasma-derived exosomal RNAs by deep sequencing. They obtained a total of 101.8 million raw single-end reads from 14 size-selected sequencing libraries and detected significant fractions of RNA species including rRNA (9.16 % of all mappable counts), lncRNA (3.36 %), piRNA (1.31 %), tRNA (1.24 %), snRNA (0.18 %) and small nucleolar RNA (0.01 %). However, the dominant RNA fraction was miRNA that accounted for over 42.32 % of all raw reads and 76.20 % of all mappable reads. The five most common of the 593 miRNAs detected were miR-99a-5p, miR-128, miR-124-3p, miR-22-3p and miR-99b-5p. Collectively, these accounted for 48.99 % of all mappable miRNA sequences. A further 185 potential miRNA candidates were predicted (see also Sect. 11).

Virtosomes

RNA has been found to be present in virtosomes. Experimental data indicate that it is synthesized prior to the complex leaving the cell, possibly involving the DNA-dependent RNA polymerase associated with the complex [9, 12, 15, 16, 84, 85].

Mitochondria

mitRNA was also reported to be present in plasma there being no significant relation between mitRNA in plasma and patient age ($n = 69$; $r = -0.16$, $P = 0.19$ for mitRNA). However, in a study on prostate cancer patients, non-survivors were found to have a 3.8-fold increase in mitcfRNA compared with survivors ($P = 0.003$; non-survivors: median copies, 16,038; interquartile range, 5,097–48,544 copies; survivors: median copies, 4,183; interquartile range, 2,269–8,579 copies) [47].

Parasite RNA Release

As with parasite DNA, this is a little studied area, but the possibility of parasite nucleic acids being present as cfRNA exists [64].

4 Mechanisms of Exit from and Entry into Cells by cfDNA and cfRNA

4.1 Mechanisms for the Exit of cfDNA and cfRNA from Cells

It is clear that cfDNA and cfRNA are present in blood in various forms, but how they leave affected cells and enter the blood stream has not been well investigated. It is assumed that apoptotic and necrotic fragments, especially from tumors, could be released directly into the blood stream. Garcia Olmo et al. [86] considered the possible release mechanisms, but came to no specific conclusion concerning the presence of higher cfDNA levels, especially of non-mutated cfDNA significantly elevated at the early stages of tumor progression.

Release of LINE-1 from both HeLa and HUVEC cells, in vitro, could be reduced by treating the cells with inhibitors of protein secretion [87]. Thus, DNA release from HUVEC cells was reduced by 30, 35 and 19 % for monensin, glyburide and methylamine, respectively. However, monensin reduced DNA release from HeLa cells by only 15 % while glyburide actually increased cell-surface bound DNA by 50 %.

Exosome formation is a more evident process involving exocytosis of the membrane bound cfDNA and cfRNA. However, the picture for virtosomes is not so clear in that although the complex is formed with lipo-protein they do not appear to have a standard limiting membrane [88, 89]. The virtosomal release process is energy dependent [10].

4.2 *Mechanisms for the Entry of cfDNA and cfRNA into Cells*

When considering the mechanisms by which cfCNAs are taken up, two aspects need addressing: (i) entry into the cytoplasm with the avoidance of cfNA destruction and (ii) passage from the cytoplasm into the nucleus where it may act either epigenetically or be incorporated into a chromosome.

Cytoplasmatic Entry

Early studies on the uptake of DNA and RNA into cells have been reviewed by Stroun et al. [37]. However, subsequently, there have been few studies on the mechanisms of cfRNA cell uptake. Since most cfRNA release so far recorded tends to be via exosomes (Table 3), it is very likely that cytoplasmic entry will be by a form of endocytosis. This is supported by studies on plant siRNA in which the endolysosomal system is considered to be involved [93] while in Drosophila cells, dsRNA uptake from the environment requires receptor-mediated endocytosis [94]. Lee et al. [95] have linked gene silencing by miRNAs and siRNAs to endosomal trafficking. In the few RNA uptake studies available, caveoli are prominent showing no hydrolase activity (Table 3).

It should be noted that dsRNA entry into murine GEnC cells required complex formation with cationic lipids for entry via clathrin-dependent endocytosis though it was independent of endosomal acidification [90].

Although Rh110-labelled siRNA phosphorothioate (PTO)-modified ON (TM6-6) entered into ECV304 cells [96], similar results could not be obtained with either the human T-lymphoma cell line Jurkat 17 [97] or the mouse B-lymphoid cell line BJA-B [98]. In the absence of PTOs, the uptake of the siRNA was reduced and the molecules distributed throughout the cytoplasm [99].

More recently, mechanisms of cfDNA entry have been demonstrated including the entry of various sources of bacterial DNA and mitDNA through the Toll receptor system [100–105]. Nevertheless, current research indicates that cfDNA can enter cells by various routes (Table 4). The mechanisms involved in this cell entry process include endosomes, caveoli and T-tubules. However, the mechanism by which the cfDNA avoids the digestive processes of the lysosomal system is not clear. The uptake of naked plasmid DNA via endosomes has been demonstrated to block endosomal acidification resulting in the lack of activation of the hydrolases present and hence DNA digestion [108]. Such DNA was considered by these authors to remain in the endosomes and to move to the nuclear membrane where it could be transferred directly into the nucleus.

Caveoli have different endocytotic functions from those of the clathrin-coated pit pathway. Ligands bound to receptors that are internalized by caveolae can be delivered to four different locations in the cell. At least four different caveolae membrane traffic patterns are distinguishable during potocytosis so offering a

Table 3 Possible mechanisms of uptake of cfRNA for a variety of cell types

Cell	RNA	Vehicle	References
Murine GEnC	dsRNA	Endosomes	Hägele et al. [90]
ECV304	siRNA phosphorothioate	Caveoli	Fra et al. [91]
2B2318 lymphocytes	SFV-VIP21 virus (dog/simian)	Caveoli	Fra et al. [92]

Table 4 Possible mechanisms of uptake of cfDNA for a variety of cell types

Cell/tissue	DNA	Vehicle	References
Leukocytes	Bacterial DNA; mitDNA	Toll system	Chuang et al. [101], Hemmi et al. [105], Cornélie et al. [102], Barton et al. [100], Dalpke et al. [103] and El Kebir et al. [104]
Myofibres	Plasmid DNA; mRNA	Caveoli; T-tubules	Wolff et al. [106]
Human keratinocytes	Plasmid DNA	Macropinocytosis	Basner-Tschakarjan et al. [107]
J77 cells	Plasmid DNA	Endosomes	Trombone et al. [108]
Murine GEnC cells	dsRNA	Endosomes	Hägele et al. [90]
Human MCF7 breast cancer cells	Human chromatin fragments	Endosomes	Yakubov et al. [109]
NIH3T3	kras	Exosome	García-Olmo et al. [86]

mechanism for bypassing the lysosomes [110, 111]. However, there is still the possibility that the caveoli may eventually link with the lysosomal system [112, 113]. It is not clear how the RNA escapes from the either endosomes or the caveoli, but it is able to exert a biological effect upon the recipient cells [81, 90].

Thus, it is possible that cfDNA can enter primarily via either macropinocytosis into cells in vitro or via caveoli into muscle in vivo and block the acidification of the endosomes so preventing their development into lysosomes and hence degradation of the DNA. In addition, the DNA can exploit the endosomal movement to the nucleus so transporting the cfDNA prior to its release and entry into the nucleus. An example of such activity concerns exosomal mutated cfDNA from a colorectal cancer patient transforming NIH3T3 cells that were able to initiate tumors in rats [114, 115].

It has been suggested that DNA binding proteins are involved in the uptake of cfDNA. The studies of Basner-Tschakarjan et al. [107] indicated that ezrin and moesin are functionally associated with some transmembrane receptors such as the EGF, CD44 or ICAM-1 receptor. These workers considered that these binding proteins were important in the uptake of plasmid DNA into keratinocytes. Subsequently, there has been little information on such binding proteins and alternative mechanisms have been proposed.

Histones H1 [116–121], H2A [122–124], and H3 and H4 [125, 126] have been shown to be effective mediators of transfection. The postulated mechanisms by

which histone H1 increases gene transfection are through DNA condensation and DNase protection. DNA delivery activity may be mediated by two mechanisms, namely, electrostatically driven DNA binding and condensation by histone and nuclear import of these histone H2A · DNA polyplexes via nuclear localization signals in the protein [116]. It is also possible that, because histones can increase the permeability of membranes by ionic interaction, this mechanism could aid complexes such nucleosomes to enter recipient cells [127].

Virtosomes might also enter cells by one of the mechanisms described above [88]. Being comprised of DNA, RNA and glycolipoprotein and failing to either pick up or lose membrane material on either leaving or entering cells, it is possible that a mechanism similar to that exploited by histones could lead to the direct uptake of virtosomes through an ionic interaction between a part of the glycolipoprotein present and the cell membrane. This proposition is also supported by Wittrup et al. [128] who demonstrated that naked plasmid DNA uptake occurred via proteoglycan dependent macropinocytosis.

cfCNA Entry into the Nucleus

The nuclear membrane presents a considerable barrier to the entry of nucleic acids with the nuclear pores permitting a passive transport limit of 70 kDa molecular mass or ~10 nm diameter [129]. Nevertheless, DNA can be seen to enter the nucleus of chick embryo fibroblasts [54, 130], HeLa cells [130], L29 mouse fibroblasts and Krebs 2 ascites carcinoma cells [109] as well as plant nuclei (reviewed in [131]). The mechanism by which DNA enters the nucleus is not clear. It is known that, for mediated active transport through the nuclear pore complex, nuclear proteins require a nuclear localization signal that contains basic amino acids and can be recognized by cytosolic factors [132]. For this to occur, the nuclear pore can expand to approximately 30 nm [133]. This can be shown to function experimentally on coupling 100 nuclear localization signal peptides/kilobase pair of DNA for the nuclear delivery of the DNA [133, 134]. It is important to remember that the mediation of nuclear import of DNA is aided by the presence of H1 histone as seen with gene transfection.

Specific proteins appear to be involved during RNA movement from cytoplasm to nucleus. Thus, siRNAs need to be linked to an argonaut protein for transfer to the nucleus as in the case of NRDE-3 in *Caenorhabditis elegans* [135].

4.3 Conclusions

cfCNAs are present in a variety of forms that are capable of entering cells with which they come into contact. The mechanisms of entry, and in some cases exit, have still not been fully elucidated for either cfRNA or cfDNA although caveoli and pinocytosis seem to be implicated. As yet, there is no information as to the possible

rôles that connexins, innexins and pannexins might play in the intercellular movement of nucleic acids [136, 137].

Both naked DNA and virtosomes released into the blood can move to other parts of the organism and into host cells. On entering cells of a similar type no obvious effects occur.

However, if the uptake is into cells of a different type, the biological activity of the host cell may be modified [58]. The uptake of cfDNA by stem cells raises interesting possibilities [138]. The modification of a cell's biology on the receipt of tumor cell cfDNA has particular implications for the formation of metastases through both the release of cfDNA from tumor cells into the circulation and the ability of the cfDNA to move to cells in other parts of the body. In particular, and in spite of the blood brain barrier, cfDNA can move to the amphibian brain [139] and the human maternal brain [140] whilst fetal cells can move to the female mouse brain [141].

cfRNA in its various forms can behave similarly and in a few cases has been shown to modify the biology of the host cell though there are few studies currently available on this aspect of cfRNA.

In view of the fact that both cfDNA and cfRNA have been implicated in tumor induction, maybe the question should be asked "Should blood collected for blood transfusions be screened for specific forms of cfNAs prior to use?"

5 Mitochondrial Release and Uptake of cfNAs

There is not much known about the release of mitcfDNA from animal mitochondria, other than through either damage or cell death. Release of mitcfDNA from such mitochondria may be a key link between trauma, inflammation and systemic inflammatory response syndrome [142]. It has also been demonstrated that when mitochondria are damaged by external hemodynamic stress, they are degraded by the autophagy in cardiomyocytes. The mitcfDNA that escapes from the autophagic vesicles can lead to Toll-like receptor 9-mediated inflammatory responses in cardiomyocytes that is capable of inducing myocarditis and dilated cardiomyopathy [143]. In plants, a mitochondrial permeability transition pore complex exists together with the inner mitochondrial membrane so permitting the passage of molecules of $<$1,500 Da, [144]. However, currently there is no evidence for a similar passage of DNA in animal/human mitochondria. Nevertheless, studies by Ibrahim et al. [145] on isolated mitochondria from a range of organisms demonstrated that the efficiency of mitochondrial uptake depends on the sequence of the DNA to be translocated becoming sequence-selective for large DNA substrates. ATP needed to be hydrolyzed in order to enhance DNA import. The presence of ATP also allowed tight integration of the exogenous DNA into mitochondrial nucleoids [145].

6 cfNAs Can Enter and Express in Other Cells

6.1 cfDNA

In vitro studies on mammalian cells have shown cfDNA uptake by recipient cells and its subsequent expression. This may be due to either epigenetic or genetic responses.

Immune Response

An allogenic T-B lymphocyte co-operation involving lymphocyte subsets from human donors with different allotypes was studied. B lymphocytes cultured in the presence of the supernatant from the culture medium of T cells, previously exposed to inactivated herpes simplex virus, were able to synthesize an anti-herpetic antibody with some allotypic markers of the T cell donor. The same effect on B lymphocytes was found both with DNA purified from the supernatant of the T cell culture medium and the non-ultra-centrifuged supernatant [146].

Anker et al. [147] also used nude mice injected with DNA extracted from the complex released by human T lymphocytes previously exposed to inactivated herpes or polio viruses. Tested for its neutralizing activity by human anti-allotype sera, the serum from these mice showed synthesis of anti-herpetic or anti-polio antibodies depending on the antigen used to sensitize the T cells. This showed the antibodies to carry human allotypes. Moreover, on concentration, the newly synthesized complex transformed much more efficiently than did either the DNA purified from the supernatant or the crude supernatant itself.

Effects on Cell Division

The DNA released from mouse tumor cell lines J774 cells (leukemia) and P497 cells (glial tumor), as well as non-stimulated lymphocytes, was isolated from the culture medium by ultracentrifugation and agarose gel chromatography [58]. After concentration, the released DNAs were added to the culture media of each of the different cell types. Thus, the tumor cell lines were each incubated in the presence of either of the two tumor cell line DNAs or the lymphocyte DNA i.e. each cell type was incubated in the presence of either a self DNA or each of two foreign DNAs. ^{3}H-thymidine was added to the cultures of each cell type together with the particular donor DNA and the amount of nuclear incorporation of the ^{3}H-thymidine into DNA of the recipient cells was measured (the index of DNA synthesis). The levels of DNA synthesis in the tumor cell lines was the same in the presence of either of the DNAs released by the two tumor cell lines, but was reduced by about 60 % in the presence of the lymphocyte DNA [58]. Conversely, the incubation of the non-stimulated lymphocytes in the presence of either of the tumor cell line DNAs

showed an initiation of DNA synthesis in the presence of the tumor DNAs, but not in the presence of the lymphocyte DNA [58].

Viola-Magni et al. [148] demonstrated that similar reciprocal events occur between stimulated and non-stimulated lymphocytes.

cfDNA Effects in Irradiated Cells – By-Stander Effect

Exposure of Chinese hamster ovarian cells to X-rays at an adaptation dose of 10 cGys led to a transposition of the chromosomal peri-centromeric loci of homologous chromosomes from the peri-membrane sites to approach each other and an accompanying activation of the chromosomal nucleolar-forming regions [149]. The movement of the peri-centric loci appears to be associated with repair of the DNA double-strand breaks during the development of an adaptive response to radiation. Growing untreated cells in medium containing DNA fragments isolated from the medium of treated cells led to their exhibiting similar changes to those seen in the treated cells. Incubation of the untreated cells in medium containing the DNA fragments from medium of untreated cells had no such effect [149]. This is known as the radiation–induced by-stander effect and can be seen both in vitro, as described above, and in vivo [150–155].

cfDNA Effects on Myocardiocyte Contraction Rates

cfDNA was isolated from patients with myocardial infarction followed by the separation of AT-rich fragments of the human satellite 3 tandem repeat (1q12 region) and GC-rich fragments of the rDNA [156]. When fed in vitro to neonatal rat ventricular myocytes in culture, AT-rich fragments (1 ng mL^{-1}) increased the frequency of cardiomyocyte contractions whilst GC-rich fragments (0.5 ng mL^{-1}) decreased the contraction frequency. Serum cfDNA from patients with acute myocardial infarction decreased contraction frequency in proportion to the cfrDNA content so implying that the GC-rich cfrDNA circulating in the blood myocardial infarct patients might affect the contractile function of the myocardial cells [156].

Tumor Induction

The SW 480 cell line, originating from a human colon carcinoma, contains a point mutation of the K-ras gene on both alleles. These cells in culture released DNA containing the mutated K-ras gene. When NIH-3T3 cells were directly cultured in the presence of non-purified SW 480 cell culture supernatant, transformed foci appeared in similar numbers to those occurring after a transfection using a cloned K-RAS gene administered as a calcium precipitate [157].

The effects on cultured cells of plasma from healthy individuals and patients with colon cancer were also determined. NIH-3T3 cells and human adipose-derived

stem cells (hASCs) cultures were supplemented with plasma cfDNA from either patients with K-RAS-mutated colorectal tumors or from healthy subjects by either (i) direct addition of plasma to cultures in standard plates or (ii) avoiding plasma-cell contact by filtering through membranes (0.4 μm pores) placed between the plasma and the cells. No K-RAS mutated sequences were detected in hASC cells by qPCR. However, human gene transfer occurred in most cultures of NIH-3T3 cells, since they were shown to contain sequences for human K-ras, p53 and ß-globin. NIH-3T3 cells were oncogenically transformed after being cultured with plasma from colon cancer patients, confirmed by carcinoma development in NOD-SCID mice after injection with the transformed NIH-3T3 cells. The human mutated K-RAS sequence was also found free in the mouse blood. The presence of the artificial membrane between the NIH-3T3 cells and the tumor patient plasma gave similar results showing that the transforming factor had a diameter of less than 0.4 μm. A TEM study of the plasma fraction passing through the membrane pores confirmed the complete absence of cells but the presence of vesicles $<$0.4 μm diameter [114]. These were later shown to be DNA containing exosomes [50].

Trejo-Becerril et al. [115] reported a similar set of results using the same experimental system as that used by Garcia-Olmo et al. [114]. In further experiments, Garcia-Olmo et al. [158] showed that plasma K-RAS cfDNA was found during a 2-year period following surgical removal of the colorectal tumor from patients. This DNA also yielded similar results with the same experimental system involving NIH-3T3 cells and NOD-SCID mice [158].

RAR2 gene methylated cfDNA uptake into HeLa and human umbilical vein endothelial cells was twice as efficient as that of unmethylated cfDNA. Hypermethylation is a common alteration of tumor related cfDNA from cancer patients and as methylated *RAR 2* gene cfDNA is more prevalent than the unmethylated form in intracellular traffic, it is considered they pose a higher transformation potential [159].

Clearly, cfDNA released from healthy cells can move to other parts of the organism and into host cells. Entry to cells of a similar physiohistological type does not lead to a changed biological activity. However, if the uptake is into cells of a different physiohistological type, a changed biological activity in the host cell may occur. The above results have implications for (a) the formation of metastases by DNA released from tumor cells into the circulation termed "genometastasis" [114, 138, 158, 160–162] and (b) the ability of cfDNA to move to cells in other parts of the body, including the brain, in spite of the blood brain barrier, where it could be taken up and expressed [14].

Gene Replacement Therapy

Based upon data indicating that chromatin fragments possessing recombinagenic free ends were present in the plasma and serum (section "Tumor Induction"), it was possible to exploit them in gene replacement therapy. Small fragments prepared from human chromatin from non-mutant cells were added to the culture medium of

human breast cancer cells having a 47-bp deletion in the *CASP 3* gene. The restoration of caspase 3 activity occurred in 30 % of the treated cells [163].

6.2 cfRNA

Few studies have been performed on the entry of cfRNA into other cells. However, dsRNA polyriboinosinic polyribocytidylic acid (polyI:C) activated murine glomerular endothelial cells via RIG-1 in the cytosol to produce inflammatory cytokines, chemokines and type I interferons [90].

The experimental delivery of siRNA is very difficult and so it possible that the natural uptake of sicfRNA will be minimal. However, when entry of siRNA has been demonstrated, there is an apparent dose-dependent siRNA-mediated suppression of lamin A/C in primary human umbilical vein endothelial cells [96, 99]. Perhaps more importantly, Skog et al. [81] showed that mRNA and miRNA can be taken up by normal host cells including brain and microvascular endothelial cells. In addition, RNA-containing glioblastoma derived vesicles were shown to stimulate proliferation of a human glioblastoma cell line.

7 Can cfNAs Influence the F1 Generation?

This question was recently considered since cfNAs have been demonstrated to be capable of modifying cells into which they enter [64]. The continual circulation of cfNAs around the organism may result in their entry into adjacent tissues. If the cfNA is not mutated, then any entrance and expression would not necessarily be detectable. However, as has already been considered for mutated cfNAs, the likelihood of the development of metastases is a possibility [158]. Foreign cfNAs could also circulate though there has been little evidence that this is a major problem in the normal individual. Nevertheless, if such cfNA could enter the gonads, then this could offer a mechanism for horizontal gene transfer (HGT) to operate, HGT involving the movement of genes from one organism to another. Although HGT has been identified to occur in lower organisms such as bdelloid rotifers [164] or Lepidoptera [165] currently, there is no such evidence for eutherians including man. This is likely to be prevented through the presence of the distinct germ cell line being separate from the soma [166]. This would appear to act through the difficulty of the natural entry of cfNAs into mammalian sperm and ova to form a modified zygote [64, 167].

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