Pyknotic chromatin in mitonucleons elevating in syncytia undergo karyorhhexis and karyolysis before coalescing into an irregular chromatin mass: Differentiation of Epithelial Domes, Part 2

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Abstract

Pyknosis, karyorrhexis and karyolysis, harbingers of programmed cell death in many systems, appear to be driving forces that transform Ishikawa monolayer epithelial cells into differentiated dome cells. The heterochromatin affected by these process is contained in multiple nuclei aggregated in the syncytia that form when Ishikawa monolayers are stimulated to differentiate (Fleming, 2016a). The nuclear aggregates are enveloped in a double membrane staining for the endogenous biotin in mitochondrial carboxylases. The structure called a mitonucleon becomes vacuolated, along with the heterochromatin it envelops, and this structure elevates with the apical membrane of the syncytium 6 to 8 hours into the 20 hour differentiation, becoming increasingly pyknotic. This phase of the differentiation comes to an end when the mitonucleon membranes are breached and nuclei emerging from the aggregated state can be seen to fragment explosively. Fragmented DNA associates with an array of microtubules, filling the large central clearing of the predome. Some chromatin remains unfragmented and can be seen of the edges of the predome clearing. Cell death does not occur. Instead, the fragmented DNA coalesces into an irregular mass within the apical and basal membranes of the predome under which fluid has been accumulating. From the chromatin sheet, nuclei emerge amitotically as described in Part 3 of this series (Fleming, 2016c)

Introduction

For fertile women, *the* uterine endometrium undergoes differentiation throughout most of the 28 day menstrual cycle, preparing for the complex process of implanting a blastocyst. Changes include elaboration of glands whose secretions are essential to the process. In what may be apoptosis on a grand scale, the differentiated tissue is shed if fertilization does not occur. Using Ishikawa endometrial epithelial cells that contain steroid receptors (Nishida et al. 1985), we were able to elicit *in-vitro* formation of gland-like structures that begin as domes, hemispheres of cells enclosing fluid (Fleming, 1995). The domes form in an approximately 20 hour period by a sequence of structural changes noteworthy for the appearance of cells with “optically clear nuclei” and “signet-ring” cells. These unusual structures have been identified in adenocarcinoma in various organs as well as in non-cancerous tissue as reviewed in the accompanying paper(Fleming, 2016a).

The earliest event in the differentiation is the formation of syncytia in which nuclei from undifferentiated monolayer cells aggregate. These aggregates become enveloped by a double membrane staining for the endogenous biotin of carboxylases characteristic of mitochondria. Small vacuoles or bubbles developing in the heterochromatin of mitonucleons result in “optically clear” nuclei (Fleming, 2016a) A

single large vacuole is generated within the double membrane forming the border of the mitonucleon. When fixed and stained, the apical elevation flattens so that the mitonucleon takes on the appearance of a "signet ring cell," another structure found in cross sections of tissue from some cancers (Fleming, 2016a).The "signet stone" of such a structure, made up the heterochromatin of aggregated nuclei, becomes quite pyknotic as it is pressed against the syncytial apical membrane pushed up by the developing central vacuole.

Vacuole formation in mitonucleons appears to be essential for predome elevation, a process that occurs during the first third of the differentiation (Fleming, 2015a). The next stage of differentiation is signaled by disruption of the mitonucleon membranes, perhaps when the pressure becomes too great, ushering in the next significant structural change. The multiple aggregated nuclei previously packaged in the mitonucleon disaggregate to some extent and chromatin deconstruction begins. Chromatin granules are forcefully generated from nuclei, eventually filling the entire syncytial structure and becoming filamentous as they attach to microtubule-like structures. Barely visible filaments weakly staining for hematoxylin fill the predome for a brief period of time. The coalescence of those fibrils signals the start of the last phase of differentiation. A swath of hematoxylin stainable material becomes visible between apical membrane protrusions from which nuclei emerge to fill the apical-basal envelope of the syncytium now stretched across the cavity of the newly formed dome. In essence, in differentiating Ishikawa cells, the DNA undergoes dramatic changes but is not destroyed. As will be discussed, chromatin is recycled amitotically, almost certainly following changes relevant to differentiation.(Fleming 2016c)

Results

As described in the accompanying paper (Fleming 2016a), mitonucleons generated by the elaboration of biotin-containing membranes around nuclear aggregates are transient structures involved in predome elevation. Typically, a predome contains four to six mitonucleons, each the result of the aggregation of 4 to 8 nuclei within the multinucleated syncytium.

Stages undergone by mitonucleons can be seen in the elevated living predome in fig. 1a. The multiple bubble-like clearings within heterochromatin, characteristic of cancer cells with "optically clear" or "ground glass" nuclei, (Fleming, 2016a) can be seen in each of the mitonucleons. At one o'clock, it is possible to make out a protruding membrane formed as a result of a large vacuole within the mitonucleon that is flattening nuclear material against the apical membrane. (Fleming 2016a) Vacuole-generated protrusions in predomes are deflated to some extent upon fixing

and staining (fig. 1b), resulting in a profile more analogous to two dimensional sectioned tissue and very similar to that of "signet ring cells" observed in such sections from a variety of cancers (Fleming, 2016a). The mitonucleons at twelve o'clock and five o'clock appear to be at an earlier stage prior to the generation of a large, central vacuole, although such a vacuole seems to have begun pushing up on the heterochromatin in the mitonucleon at 5 o'clock. The mitonucleon in the center of the predome appears to be at a later stage, with the heterochromatin no longer in a tight pyknotic structure, but spread through the apical membrane around the base of membrane protrusions. A relatively rapid progression through these stages is suggested by the fact that they can be found together in a predome.

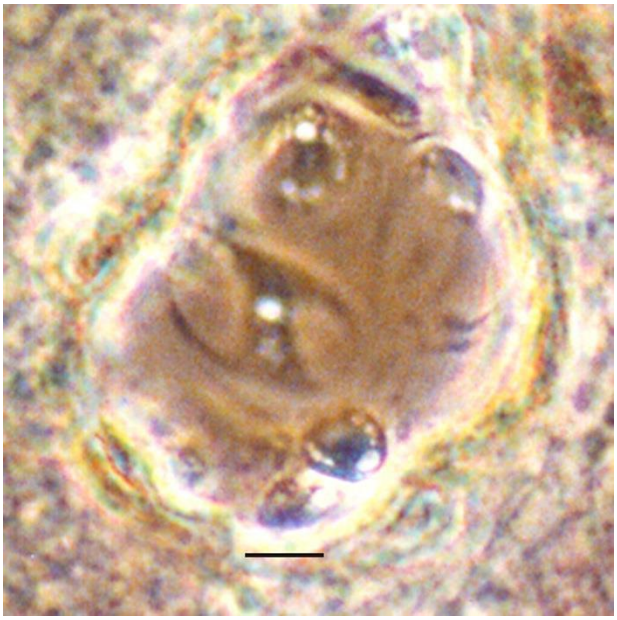

Fig. 1a Mitonucleons in a predome, living culture of differentiating Ishikawa cells.

Opaque heterochromatin in mitonucleons can be seen at three different stages in elevating predome.

Bar=50 microns

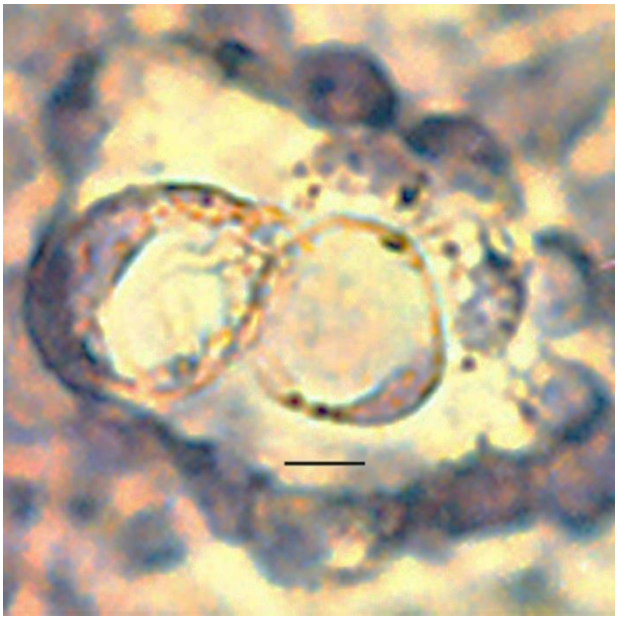

Fig. 1b Fixed and stained predome with mitonucleons resembling signet ring cells

Large central vacuoles are a prominent feature of both mitonucleons in this predome fixed and stained with hematoxylin and eosin as well as with antibody to biotin.
Bar=50 microns

The vacuoles form rapidly (one to two hours) and appear to generate pressure and to bring about significant elevation of apical membranes of the predome. The vacuoles deflate upon fixation supporting the suspicion that they arise from the generation and containment of a gas such as CO2 that is readily dissipated upon fixation (Fleming,

2016a). Particulate structures slightly larger than nucleoli accompany the excessively pyknotic heterochromatin in fig. 1b, along with particulate pigmented material within the heterochromatin.

Figure 2a shows a fixed and stained predome as the transient mitonucleons begin to come apart. The structure on the left in fig. 2a looks like a typical "signet ring" cell except that the compressed aggregated nuclei (the disc in fig. 1b shows how extreme that compression can be) appears to disaggregating, so that at least three disc-shaped nuclei can be detected. Disassembly of the mitonucleon in the neighboring protrusion on the right in fig. 2a is further along. The double membrane has been breached so that the dark structures a little larger than nucleoli accompanying the elevating heterochromatin are leaking back into the syncytial cytoplasm (arrow). Chromatin in that second mitonucleon is no longer pyknotic and stains less intensely with hematoxylin.

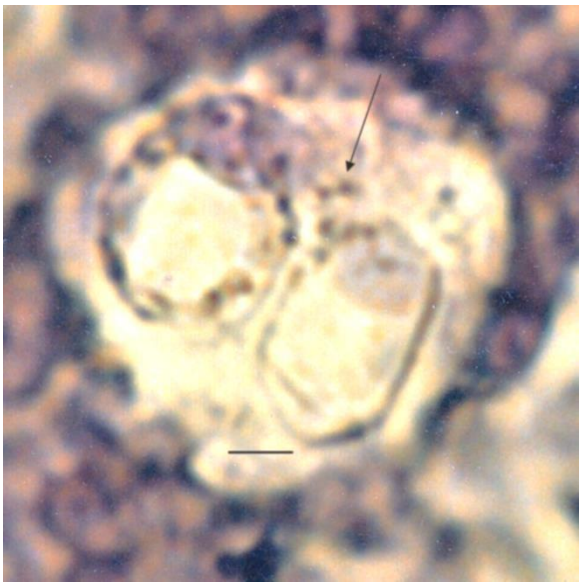

Fig.2a Structural changes in mitonucleons as the biotin containing membranes of the mitonucleon begin to dissolve.
Bar = 50 micron

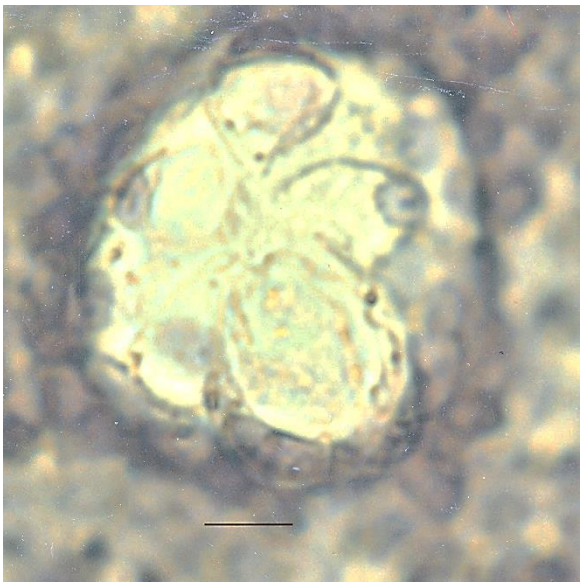

Fig. 2b Further loss of biotin containing membranes that defined mitonucleons.

Bar = 50 micron

In the predome shown in fig. 2b the differentiation is further along. The "remains" of at least five mitonucleons can be detected as apical membrane protrusions. The heterochromatin in what is left of the mitonucleons is in varying stages of "fading." Ovoid but pale stained structures can be seen in the protrusions at 10 o'clock and at 2 o'clock. The irregularity of the stain in the protrusion at 7 o'clock suggests

disaggregation of nuclei. The protrusions at 12 o'clock and 5 o'clock are furthest along. A very faint blue-violet stain can be seen spreading across the middle of the protrusion at 12 o'clock . In the largest protrusion at 5 o'clock, no hematoxylin stain can be detected, but fibrous material can be seen through the apical membrane.

Diminished hematoxylin uptake signals changes in the physical characteristics of chromatin. Such a change sometimes called karyolysis is usually cited in the literature along with karyorrhexis, the fragmentation of nuclear material, as harbingers of programmed cell death. These processes follow rapidly on each other during Ishikawa differentiation, as chromatin, previously in mitonucleons, is deconstructed.

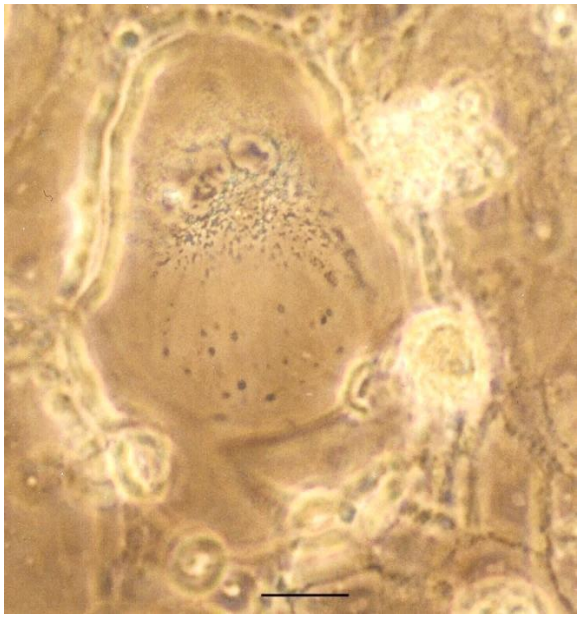

Fig. 3a Aggregated nuclei fragmenting in apical-basal predome envelope.

Microtubule-like structures deploy along the long axis of the apical membrane protrusion. Bar=50 microns

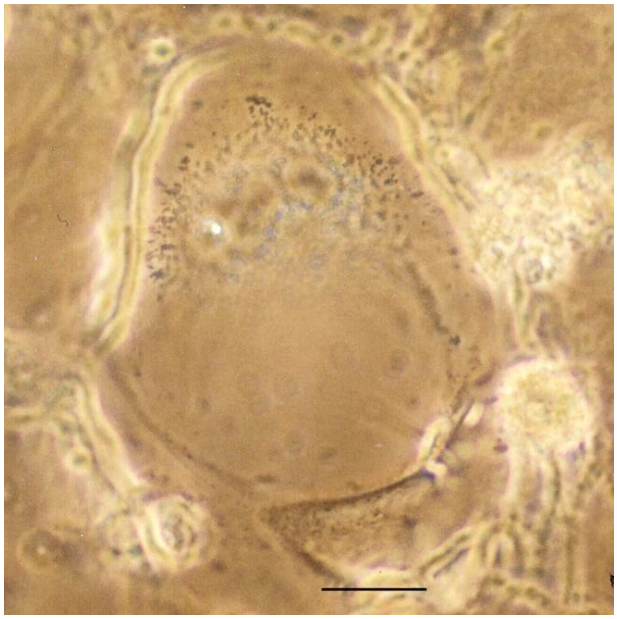

Fig. 3b Fragments from nuclei throughout the spread throughout the apical-basal predome envelope.
Same structure as shown in fig. 3a, focusing down on granular material "exploding" from disaggregated nuclei. Bar = 50 microns

Disaggregation of nuclei as discussed for fig. 2a is more extensive in the predome in fig. 3, but the much more dramatic change is that granular fragments appear to be "exploding" in all directions from these nuclei, and a spindle-like array of what appears to be microtubules has deployed. This material has not been fixed so the picture is of a living predome. Whether focusing on the top of the apical-basal

predome envelope or down into that envelope, granular fragments initially appear to be flying in all directions from the nuclear remnants. Additionally a structure that looks similar to a spindle can be observed to have deployed in fig. 3a, perhaps from the centriole-like structures so obviously accompanying mitonucleons in fig. 2a.

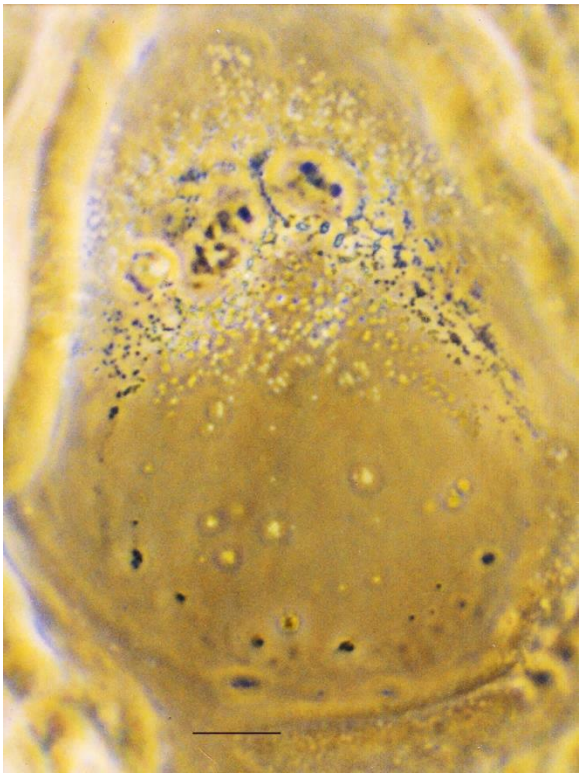

Fig. 4 Fragments from disaggregated nuclei moving to the opposite pole of the predome along microtubule-like structures

Bar=25 microns

Fig. 4 is a picture of the exploding nuclei at a higher magnification taken minutes after the pictures in fig. 3. Granules can still be detected above and below the fragmenting nuclei. Many granules appear to be associating with the spindle-like structures and moving toward the opposite pole, so that these structures resemble in form and function the mitotic spindles that pull chromosomes apart. In this instance the tubule-like structures appear to be organizing the spread of highly fragmented chromatin material. Perhaps it is both the fragmentation and the spread of chromatic material, together with changes in association with proteins, that explains the resulting diminishment of hematoxylin uptake

Fig. 5a is a predome characterized by parallel filaments of varying thicknesses, a light micrograph, most probably representing (and therefore visible by light microscopy) the largest of the quasi-parallel array of chromatin fibers previously found in

endometrial tissue and in cancers. The thickest of these fibers may represent the beginning of coalescence of the chromatin. Fig. 5b is a fixed and stained predome

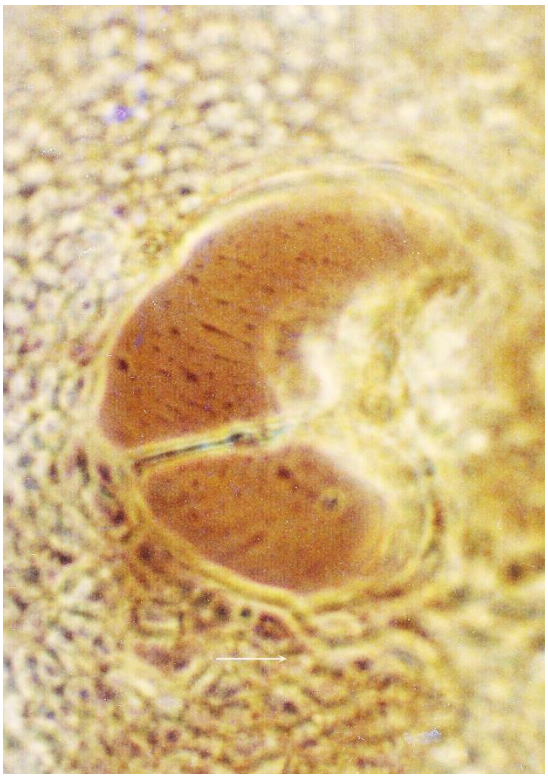

Fig.5a Living predome with filaments of varying thickness arrayed more or less parallel to each other throughout membrane envelope.
Bar=25um

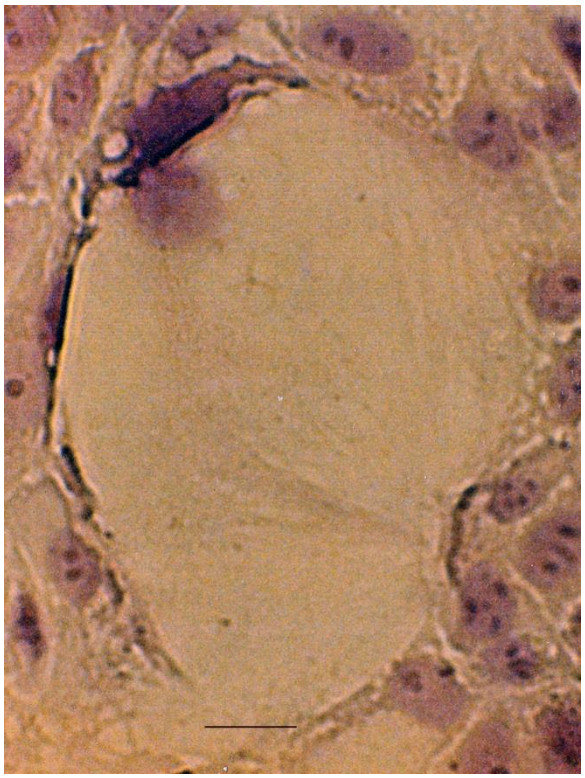

Fig. 5b "Predome clearing" fixed and stained with hematoxylin. Barely visible threads can just be made out stretching from pole to pole. Heterochromatin can be seen on the edges and at the poles.
Bar=25um

observed at approximately the same time in the process of differentiation. Heterochromatin can be observed at both poles of the structure, and, it is just possible to detect threads staining for hematoxylin, stretching from one pole of the predome to the other. Additionally, some lightly stained chromatin can be seen at the edge of the cell contiguous with heterochromatin.

The predome in fig. 6a was seen 13 hours after the start of differentiation and is not fixed. Nevertheless, recognizing DNA by its refractivity, a couple of surprising observations can be made. First, the structure of the DNA is extremely unusual. Linear chromatin appears to be stretched around regions of the apical membrane that

are protruding. Rounded shapes appear to be emerging within the mass of chromatin. This is the beginning of the process that will be discussed in detail in the third paper in this series, a chromatin mass can be detected, out of which ovoid nuclei begin to emerge in an amitotic process (Fleming, 2016c).

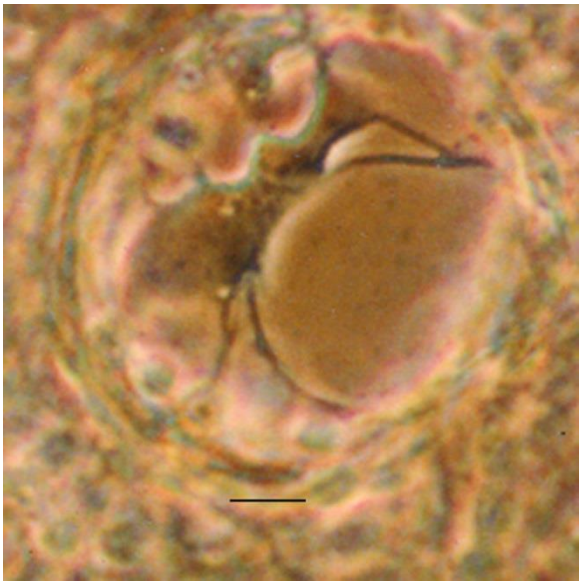

Fig. 6a A chromatin mass spreading throughout the envelope of the apical and basal membranes of the predome.

While some of the refractive mass of chromatin material looks as though it is rounding up, other material appears to be stretched around apical membrane protrusions.
Bar = 50 microns

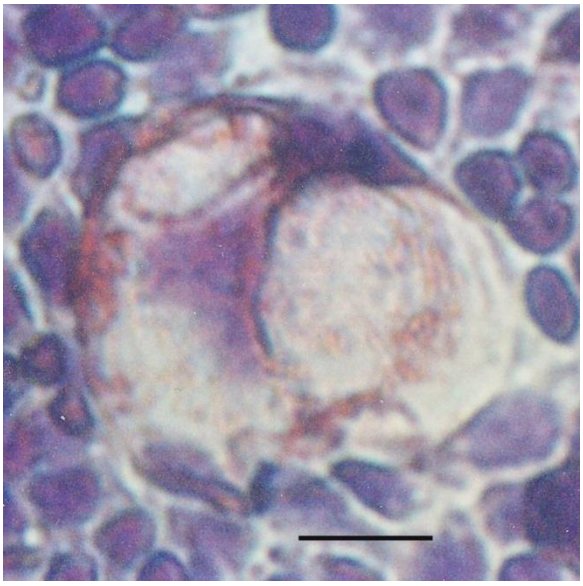

Fig 6b Fixed and stained chromatin and endogenous biotin in predome.

Collapse of protrusions reveals redistribution to apical membrane, particularly up through the protrusion
Bar = 50 microns

In the unstained predome in fig. 6a, the apical membrane protrusion is up and out of focus. A similar protrusion in the stained predome on the right has collapsed down and it is possible to detect that the protruding membranes stain for endogenous biotin. It is useful to remember that the chromatin sheet lies between the apical and basal membrane of the predome arching over a lumen that is filling with fluid. The chromatin, and it may still be filamentous, has coalesced and reacquired the characteristics that support hematoxylin staining. With the break-up of the mitonucleons, material staining for endogenous biotin appears to relocates. It can clearly be seen throughout the life of the dome associated with the dome surface, an

association that persists as long as the dome remains elevated resulting in stained domes such as those shown in fig. 6 in the first paper in this series (Fleming, 2016a)

DISCUSSION

Dramatic structural changes accompany the differentiation of Ishikawa monolayer cells into fluid-enclosing multicellular hemispheres capable of extending into tubules. Two of those transient structures appear to be particularly important. The first, initially described in 1998 (Fleming et al.1998) is the mitonucleon, aggregated nuclei from fused monolayer cells that become wrapped in a membrane containing carboxylases, apparently elaborated from mitochondria. The mitonucleon is usually formed within 6 hours after the addition of a stimulus to differentiate and enables the developing structure to move into the third dimension, up from the monolayer, through vacuole formation (Fleming, 2016a).

Additionally, there is a dramatic change in heterochromatin from hematoxylin-staining, membrane- bound, ovoid structures first into granules and then into networks of fine filamentous material, barely visible under the light microscope. An ordered array of this material spreads throughout the Ishikawa syncytium for a brief period of time approximately 10 to 12 hours after the start of differentiation.

A similar transformation from ovoid, hematoxylin-staining heterochromatin to chromatin fibers as revealed by electron microscopy (Roberts, Horbelt, and Powell, 1975) was described for human endometrium collected several days after subjects received a dose of medroxyprogesterone, an analog of progesterone. Subsequently, Mazur, Hendrickson, and Kempson (1983) found such structures in uterine endometrial tissue associated with trophoblasts, and therefore naturally exposed to hormones such as progesterone. Mazur used the term “nuclear clearing” to indicate that by light microscopy and hematoxylin staining, typical nuclei could no longer be detected. Instead, an elaborate array of fine filamentous material filling most of the clearing was revealed when sections were examined using the electron microscope.

In the decade following observations of these unusual chromatin arrays in endometrium, similar quasiparallel arrays of chromatin fibers detectable by electron micrography were observed in cancer tissue. Nakatani and his colleagues (1990) examining pulmonary tumors resembling adenocarcinomas found nuclear clearings filled with chromatin filaments, as well as optically clear nuclei associated with biotin (Nakatani et. al. 1994). Yamashita et al. (1992) found such structures in thyroid papillary cancer, importantly distinguishing them from optically clear nuclei and reporting that the clearings “appeared completely empty in hematoxylin and eosin stained sections.” Similar structures were found in pulmonary blastoma (Yang et

al.1995). Most recently, Papotti et al. (2004)) discussed "nuclear clearing due to fine chromatin" in thyroid cancer.

Each of these reports contains electron micrographs of quasiparallel chromatin fibers following one axis of a structure that is somewhat larger than the typical cell, without, of course, the boundary of the typical nuclear structure. Almost the entire clearing is filled by this ordered array of chromatin fibers, although there is still some relatively compact heterochromatin around the edges of the clearing. Furthermore with the disappearance of nuclear membranes, it must be assumed that cytosol and nuclear contents mix, similar to what happens when nuclear membranes break down during mitosis (Kutay and Hetzer, 2008). For the Ishikawa differentiation described in this paper, the term "predome clearing" has been used since the research shows that these clearings develop into domes.

"Nuclear or cell clearings," whose function remained a mystery for years, were usually observed in adenocarcinomas arising in glandular organs such as the thyroid. The results in this paper suggest that the structures may be the result of epithelial cells attempting to differentiate into tubules or gland-like structures. Optically clear nuclei and signet ring cells, structures that lead to predome clearing (Fleming, 2016a) in Ishikawa differentiation, are also frequently observed in adenocarcinomas.

There is one other example of experimental results in which DNA is found in a state similar to chromatin in nuclear clearings. Barlow and Sherman (1974) describe "large numbers of chromatin threads of uniform size intermingling throughout the nucleus, often radiating from clumps of heterochromatin" in giant trophoblast nuclei of the mouse and the rat.

In a more recent study of polypoid trophoblasts in mink, Isakova and Mead (2008) have reported that some nuclei are characterized by chromatin threads of different thickness which lie parallel to one another and frequently look beaded. This comes close to the vision of fragmented DNA shown in figs.5a and 5b, although the "beaded" look of the fibers is only seen in the unfixed, unstained predome.

Nuclear clearing resembles nuclear fading

Nuclear clearing is very like nuclear fading or karyolysis, a term appearing in the literature from early on in studies of cell death (Corper HJ, 1912). These processes are similar at least through to the "apparent dissolution" of the nucleus of a cell, as judged by the disappearance of an ovoid, hematoxylin stained structure. An array of chromatin fibers is not usually reported to accompany karyolysis, perhaps because the cell is programmed to die, whereas the comparable nuclear clearing in Ishikawa predomes, as well as in human endometrium exposed to progesterone *in vivo*, is

accompanied by deconstruction of heterochromatin into an array of filaments that fill differentiating nucleocytoplasmic structures.

During Ishikawa differentiation, filaments arise from the disaggregated pyknotic aggregated nuclei of mitonucleons in an explosive "reaction" approximately 12 to 14 hours into the process of dome differentiation. The appearance of this chromatin fiber array *in vivo* in endometrium, in response a progesterone analog and to pregnancy hormones supports a role for such structures in endometrial differentiation. It is the secretion of progesterone in vivo that is responsible for the progression of endometrium from the proliferative phase to the secretory phase, a process characterized by the elaboration of glands. And, in fact, dome differentiation in Ishikawa endometrial cells was also shown to be enhanced by the addition of progesterone, along with differentiation-inducing factor from fetal calf serum (Fleming, 1995).

DNA Fragmentation

Physical changes in chromatin usually involve the extent of chromatin condensation, as well as varying associations with proteins and, as it turns out, whether the chromatin is fragmented. Extensive arrays of chromatin fibers, more decondensed than condensed, and apparently minimally associated with protein, may insure that the DNA can be altered in whatever way necessary to bring about differentiation. Quasiparallel arrays of chromatin fibers may provide such an opportunity. On the basis of how infrequently structures such as those shown in figs. 4a and 4b were observed, it is probable that a phase in which DNA spreads out as filaments outside of the typical ovoid nucleus is relatively short-lived. Results from Ishikawa differentiation indicate that it is nuclear fragmentation (fig. 3) or karyorrhexis (a term also introduced 100 years ago) that leads to chromatin spreading as fibers.

In vitro, chromatin fragmentation has been used as an endpoint to learn about how this very large molecule is put together. Hershey and Werner (1972) demonstrated that chromatin could be fragmented to units of approximately 20 kbp (other studies have found fragments of 50kbp and higher) by alkali denaturation following proteinase digestion. On the basis of that result, these researchers suggested that genome DNA does not exist as one continuous strand but is, instead, contiguous strands held together by ribonucleoprotein Much additional research as reviewed by Nickerson (2002) suggested a model in which eukaryotic chromatin is organized into ≈30- to 150-kbp units anchored to a ribonucleoprotein-containing structure that can be broken down by exposure to proteases.

Research supporting such a model includes the observation of fragmentation of DNA in healthy, resting or proliferating cells observable in cells in agarose gels treated with

proteases in an alkaline environment (**Szabó** and Bacsó, 1995). This work, being done at the same time that interest was growing in programmed cell death, led **these researchers** to remark on the apparent similarity between the effects of apoptosis and the effects of protease treatment of nuclei, stressing that since the observation is made in otherwise healthy cells, fragmentation by itself may not always signal the onset programmed cell death.

More recently, using a clever microscopic approach of field inversion single cell gel electrophoresis, **Szabó** and his collaborators showed nuclei of healthy non-apoptotic cells (**Székvölgyi L. et al.** 2005) "exploding" into granules under the conditions of alkaline electrophoresis following proteolysis. Furthermore, their results showed that when the pH of the agarose gel was neutral, the granules stretched out into fibers. It was striking how much this otherwise unexpected phenomenon resembles what is seen happening to previously aggregated nuclei in predome syncytia at a certain point in Ishikawa dome differentiation (figs. 3a, 3b, 4a, and 4b), i.e. the apparent explosion of nuclei into fragments and subsequent appearance of filamentous chromatin material.

In vivo, an increase in chromatin fragmentation in growing cell lines deprived of serum (Solov'yan, 1997), was shown to be rapidly reversed to control levels following serum addition, an unexpected result if fragmentation is always an irreversible event in programmed death. In differentiating Ishikawa cells, the "explosive" fragmentation of nuclei is also reversible. Rather rapidly the fragmented DNA "comes back together again". It has to be assumed that the capture of fragments in linear arrays on what appears to be microtubular structures is the event that makes it possible for order to emerge from the apparent chaos of explosive fragmentation. Rearrangements, amplifications of certain regions of the genome, and the enzymatic recreation of RNA-protein linkers, along with binding to proteins, and DNA replication may also be occurring under these circumstances. Furthermore the tubules deployed as the nuclear material fragments (fig. 6) may provide the biochemical machinery necessary to move nuclei (Baker, Theurkaufund, and Schubiger, 1997), a process that will be discussed in the third paper of this series (Fleming, 2016c)

Differing Degrees of DNA Fragmentation

In addition to pyknotic nuclei, karyolysis, and karyorrhexis, one other hallmark of apoptotic cell death (Hockenberry, 1995) is the generation of double-stranded DNA fragments (as opposed to the single strand fragments we have been discussing) effected by nucleases and detected by the formation of a ladder of DNA fragments in agarose gels. Early-on in research into apoptosis, it was believed that such double stranded breaks were responsible for the compaction of DNA characteristic of the

pyknotic nuclei reported to foreshadow cell death (Liu et al., 1998). More recently Iglesias-Guimarais and colleagues (2010) demonstrated that compaction can take place even in situations in which double stranded DNA nicks/breaks do not occur. The fragmentation characteristic of single strand breaks, even without oligonucleosomal fragmentation, can also result in cells exhibiting classical apoptotic changes (Oberhammer et al. 1993; Yamaguchi et al. 2004).

Chromatin then, is subject to at least two different enzymatic processes that result in fragmentation, one involving proteases exposing naturally occurring single stranded breaks and one involving nuclease dependent double-stranded breaks, both resulting in the compaction of DNA as observed in pyknotic nuclei which can "lead to" programmed cell death or, as is being discussed in this paper, to cell differentiation. In very general terms, and possibly truistic , the least that can be said is that the processing of DNA in differentiating cells probably does not involve the loss of essential coding information, whereas, DNA fragmentation in dying cells might be expected to "go further" and to include the loss of some or all coding information.

DNA Fragmentation and Differentiation

DNA fragmentation as a part of differentiation is not unique to Ishikawa cells. An extensive literature review by N. Sjaste and T.Sjaste (2007) implicates the process of DNA fragmentation more generally in a number of different examples of differentiation. Referencing research on DNA strand breaks in differentiating nerve and muscle cells as well as fibroblasts, erythroid cells, and in epithelial cells, the authors theorize that research interest in a possible regulatory function for DNA fragmentation in differentiation was overtaken in the eighties by the increasing interest in the role of DNA fragmentation in programmed cell death. Research over the past decade supports the theory that DNA fragmentation is involved in both processes.

Furthermore, the information gained through the focus on the process of apoptosis has provided tools for research on differentiation, starting with the role of proteases called caspases, discovered because of their involvement in apoptosis (Wang et. al.1994). In ground-breaking work, Fernando et al. (2002) demonstrated that caspase-3 activity, frequently associated with apoptosis, is also actually required for skeletal muscle differentiation which it effects by activating the same nuclease responsible for apoptosis. Succinctly acknowledging the obvious paradox, these researchers entitled their paper, "Is caspase dependent apoptosis only cell differentiation taken to the extreme." In that paper, Fernando and Megeney present evidence that it is the extent of caspase 3 activation that controls the balance between differentiation and apoptosis. Fernando, Brunette and Megeney subsequently showed that endogenous caspase 3 activity is also essential for neural stem cell differentiation (2005). In another approach to, and extension of, these observations, Bulatovic et al. (2015) recently showed that sublethal activation of caspase 3

promotes differentiation of cardiomyocytes from embryonic stem cells as opposed to "lethal" activation of the enzyme which leads, of course, to apoptosis.

Additional research has shown that a second caspase, caspase 9, is also involved in muscle differentiation (Murray et al., 2008). Following up on this work, in a review, intriguingly entitled "New roles for old enzymes: killer caspases as the engine of cell behavior changes," Connolly, Jager, and Fearnhead (2014) describe 18 different caspases and their roles in a number of important cell functions in addition to programmed cell death, including differentiation programs for at least 13 different cell types. In a table, the authors describe two types of "differentiation morphology," distinguishing what they call apoptosis-like differentiation from differentiation in which apoptotic morphological changes are not observed. The review makes the point that relatively simple differentiations involving loss of the nucleus frequently present with "apoptotic morphology" whereas a second category of more complex differentiations do not.

The extreme pyknotic profile of Ishikawa nuclear aggregates as shown in fig. 1b, puts Ishikawa differentiation into the "apoptosis-like" category. But unlike other cell types in that category, the chromatin of the differentiating Ishikawa cell does not appear to be destroyed or eliminated, although it is thoroughly deconstructed into chromatin fibers following pyknosis and, in conjunction with microtubule-like structures, fills the syncytium undergoing differentiation as in fig. 4b. In fact the "fading and fragmented DNA" from pyknotic mitonucleons pulls itself back together again as shown in figs. 5a and 5b, a result that has not yet been reported for any other differentiation. This observation suggests the possibility of a third category of differentiation characterized by pyknosis with a loss in the structure of the original nuclei but in which chromatin, rather than being destroyed, is adapted for reconstitution into the cell nuclei of the differentiated state (Fleming, 2016c).

Role of Mitochondrial-like Membranes in Ishikawa Differentiation

At least one other enzyme that is not a caspase has been implicated in programmed cell death. Apoptosis inducing factor (AIF) is a mitochondrial protein that has been shown to effect the fragmentation of DNA to approximately 50 kb. (Zamzami et al. 1996; Daugas et al. 2000; Candé et.al. 2002) Furthermore, it has been demonstrated that the factor will, when added to purified HeLa nuclei, result in large scale DNA fragmentation, along with condensation of peripheral chromatin, leading researchers to posit the existence of two distinct apoptotic pathways, one involving caspases and one involving AIF (Susin et al. 2000).

By analogy with the caspases, it is possible that the pathway involving AIF in apoptosis might, if appropriately controlled, also be involved in differentiation. AIF is an intriguing candidate for the phenomenon of Ishikawa differentiation since membranes with mitochondrial characteristics are intimately associated with nuclear aggregates during the first 6 to 10 hours of differentiation providing an opportunity for the AIF, contained in mitochondrial membranes (Candé et al. 2002) to become incorporated into heterochromatin. Does AIF then fragment mitonucleon heterochromatin so that it is primed to "explode" when mitonucleon membranes break down? Could the triggering event be a short-lived elevation of the pH of the aggregate when gas is released from vacuoles during the dissolution of the mitonucleon (Fleming, 20156a)?

Whether AIF is the responsible protease, the physical evidence of exploding nuclei, suggests that a chemical process is at work in the aggregated heterochromatin before the visible evidence appears in the form of chromatin fragmentation. Szabo and his colleagues (2005) demonstrated that a transient change in pH can bring about the kind of repulsion among DNA fragments that might be suspected to be at the heart of "exploding nuclei." If not AIF, perhaps another protease becomes active when the aggregated heterochromatin is vacuolated as shown in fig. 1.

Putting Humpty Dumpty back together again

The mass of DNA in figs. 6a and 6b is as unusual as the chromatin filaments in figs. 5a and 5b, and may in fact be made up of fragments that have coalesced. All indications are that it is even more short-lived. Nuclei begin to form out of the mass in the manner described in the third paper in this series, relatively rapidly over a period of 2 to 3 hours, and by all indications, amitotically. (Fleming, 2016c)

Materials and Methods

Ishikawa cells were cultured (Fleming 1995) in phenol red-free, Minimum Essential (MEM) supplemented with 2 mM glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, and .25 mg amphotericin B (GIBCO, Grand Island, NY). The cells, obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York, were originally derived from an endometrial adenocarcinoma line developed by Nishida et al. (1985), who demonstrated the presence of receptors for both estradiol and progesterone. Cells seeded at an approximate density of 5 x 10^5 cells/cm2, were grown for 1 -2 weeks in MEM containing 5% calf serum (CS), and then transferred to medium containing 1% calf serum. Cultures left in MEM with 1% CS could survive for an additional 3-5 days with little proliferation. Assays for dome formation were done in confluent cultures, although differentiation has been observed to occur, to a limited extent, in nonconfluent cultures.

Differentiation was initiated with the addition of I0-15% fetal bovine serum (FBS). Multiple dishes were fixed and stained for biotin and./or for chromatin at different times during

differentiation. Structures were viewed using an Olympus inverted stage microscope at powers of 100X, 200X and 400X. As indicated in the text, differentiating structures were sometimes examined, and pictures taken without fixing and staining the cultures.

Other photomicrographs were taken of cells fixed by adding 4% paraformaldehyde in phosphate buffered saline (PBS) to the culture dish. After 10 min, the cells were washed gently four times with 5-10 ml PBS. A solution of 1% Triton X-100 was added to the cells to permeabilize the membrane. Again after 5 min, the culture was washed with successive changes of PBS. After washing, cells were exposed to a1:200 dilution of Extravidin-conjugated horse-radish peroxidase (HRP) (Signa) for 30 min. After further washing, a solution of 3-amino-9-ethylcarbazole (AEC), prepared by dissolving 20 mg of AEC in 2.5 ml of dimethylformamide and diluting with 47.5 ml of 50 mM potassium acetate adjusted to pH 5.0, was added to the cells together with .25% H2O2. This solution"was incubated at 37"C for 45 min to allow color to develop. The AEC solution was removed, and the cultures were examined and then stored in the presence of PBS at 4"C. If avidin linked to peroxidase is not added to the cultures, there is no reaction. If avidin without peroxidase is added first to the cultures, followed by avidin-linked to peroxidase, staining is not observed. Staining does not occur if avidin-HRP is not added to the cultures prior to AEC indicating that an endogenous peroxidase is not responsible for the staining. To ensure that avidin was reacting with biotin, we stained domes using streptavidin linked to horseradish peroxidase as well as primary antibody to biotin and secondary antibody-linked to horseradish peroxidase. Staining occurred under all circumstances, indicating that avidin does indeed react with biotin that is endogenously present in the cell in significant amounts.

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