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COMMENTARY

Putative Mechanism through Which N-Cadherin-Mediated Cell Contact Maintains Calcium Homeostasis and Thereby Prevents Ovarian Cells from Undergoing Apoptosis

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ABSTRACT. To date most of the studies involving the maintenance of ovarian cell viability have focused on the endocrine, paracrine, and autocrine factors that inhibit these cells from undergoing programmed cell death or apoptosis. Recently, studies have demonstrated that cell contact also prevents ovarian cells from dying via an apoptotic mechanism. In this commentary, the role that homophilic binding of the cell adhesion molecule, N-cadherin, plays in maintaining ovarian cell viability is presented. These studies showed that N-cadherin homophilic binding (1) is part of the mechanism through which cell contact maintains cell viability, (2) results in the activation (i.e. tyrosine phosphorylation) of the fibroblast growth factor (FGF) receptor, and (3) prevents a sustained elevation in intracellular free calcium ($[Ca^{2+}]_i$) which triggers apoptosis. These studies also revealed that hepatocyte growth factor (HGF), also known as scatter factor (SF), disrupts cell contact, which leads to a sustained increase in $[Ca^{2+}]_i$ levels and ultimately to cell death. Based on these studies, this commentary presents a putative mechanism that relates the cellular and molecular mechanism through which basic FGF, N-cadherin, and HGF/SF interact to regulate $[Ca^{2+}]_i$ levels and ultimately ovarian cell survival. BIOCHEM PHARMACOL **54**;8: 847–853, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ovary; apoptosis; N-cadherin; basic fibroblast growth factor; hepatocyte growth factor; scatter factor; intracellular calcium; cell contact

With the advent of cell culture, it was observed that dead cells lose contact with both the surface of the culture dish and other cells. As a result, dead cells are often observed floating above the viable cell monolayer. This observation is generally explained by assuming that the loss of cell contact is a consequence and not a cause of cell death. However, this concept has been challenged by several recent studies which suggest that an interaction with either extracellular matrix proteins [1] or other cells [2] plays an important role in regulating cell survival. The extracellular matrix proteins appear to mediate their action by binding to cellular adhesion molecules such as integrins [1]. This binding stimulates poorly defined signal transduction pathways, with some of the integrin-regulated pathways acting to prevent the cells from undergoing apoptosis [3, 4]. For example, the disruption of epithelial cell–matrix interactions by treatment with RGD peptides [5] or anti-integrin antibodies [6] results in apoptosis. Similarly, intracellular adhesion antibodies inhibit the interaction between follicular dendritic cells and germinal center cells and subsequently results in the apoptosis of the germinal center cells [7]. Although important to a complete understanding of how cell contact influences cell viability, the integrin-regulated signal transduction pathways will not be presented in this commentary. Rather, the reader is referred to the following reviews [1, 3, 4, 8–11].

CADHERIN-MEDIATED CELL CONTACT

In addition to extracellular matrix contact, cell-to-cell interaction influences cell survival. Cell-to-cell contact is mediated by a great diversity of cell adhesion molecules, including integrins, the immunoglobulin supergene family, selectins, and cadherins. The expression of these adhesion molecules is cell specific, with cadherins involved in mediating calcium-dependent cell-to-cell adhesion in virtually all solid tissues of multicellular organisms [12].

The cadherin superfamily can be subdivided into six gene families: classical cadherins type I (e.g. E-, N-, P-, and R-cadherin), classical cadherins type II (cadherin-6 to -12), cadherins found in desmosomes (desmocollins, desmogleins), cadherins with a short cytoplasmic domain (LT, T-cadherin), protocadherins, and distantly related gene products such as the Drosophila *fat* tumor suppressor gene [13]. The classical cadherins possess an extracellular domain that contains five tandemly arranged cadherin repeats. These repeats form four calcium binding sites. The N-terminal repeat contains the adhesive domain that is

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involved in cadherin-specific adhesions. The cytoplasmic domain is highly conserved and functions to bind catenins, cytoplasmic proteins that anchor cadherins to the cytoskeleton [14]. In addition to anchoring the cytoskeleton, there is an increasing body of evidence to suggest that cadherin is also involved in regulating signal transduction pathways [14, 15]. It is this cadherin-mediated signal transduction pathway that likely accounts for the ability of cell-to-cell contact to maintain cell viability. Given the importance of cadherins, this commentary will focus on cadherins and their role in preventing apoptosis.

CADHERIN EXPRESSION WITHIN THE OVARY

While virtually all tissues express cadherins, the mammalian ovary is an excellent model system to assess the role that cadherins play in regulating apoptosis for several reasons. First, apoptotic cell death is a major physiological event within the ovary. For example, the ovary is composed of approximately one million follicles with about 99% degenerating over the reproductive lifetime [16]. The GCs†, which comprise the follicle, undergo apoptosis as part of the physiological mechanism involved in follicular degeneration [16–18]. Second, GCs in healthy follicles are connected by both N-cadherin-mediated adhesion [19, 20] and gap junctions [20–22]. As the follicle degenerates, the frequency of both of these types of junctions decreases, and the GCs ultimately dissociate [21]. While this loss of cell contact has been documented for a long time, these relatively old observations provide a physiological rationale to investigate the relationship between N-cadherin-mediated cell contact and GC viability.

Finally, rat ovarian surface epithelial cells are a second ovarian cell type that express N-cadherin [23], are connected by both adhesion and gap junctions [24], and undergo apoptosis at the time of ovulation [25, 26]. A rat ovarian surface epithelial cell line, referred to as ROSE cells, has been developed [24]. This cell line possesses many characteristics of both normal ovarian surface epithelial cells and GCs. Therefore, ROSE cells have also been used for studies on N-cadherin-mediated cell survival.

DOES CELL CONTACT REALLY INHIBIT GC APOPTOSIS?

The initial hypothesis that cell contact regulates a mechanism that enhances GC survival is based on the observation that after culture a single GC is two times more likely to be apoptotic than a GC that has formed a cell contact [22]. However, this observation does not establish a causal relationship between cell contact and cell survival. Rather, there are several other possibilities that could account for this observation. First, this observation could be explained simply by assuming that GCs that do not form cell associations are non-viable prior to culture and, therefore, unlikely to form a cell contact *in vitro*. However, virtually all GCs are viable prior to culture. Without exposure to various survival factors such as progesterone [22] or bFGF [23], about 60% of the single GCs undergo apoptosis. The ability of progesterone and bFGF to prevent single GCs from undergoing apoptosis *in vitro* attests to their initial viability [22, 23].

A second explanation to account for the relationship between cell contact and GC viability is that aggregated GCs could produce more progesterone than single GCs. This could result in a higher progesterone concentration within GC aggregates. This concept is supported by several studies that showed that an interaction with extracellular matrix proteins enhances the ability of GCs to secrete progesterone [27, 28]. Further, estrogen secretion is greater in aggregated GCs than in dispersed GCs [29]. These observations demonstrate that aggregated GCs are more steroidogenic. However, culturing GCs in the presence of aminoglutethimide does not alter the relationship between GC apoptosis and cell contact but completely blocks progesterone synthesis [20]. This suggests that endogenous progesterone levels do not account for the enhanced viability of aggregated GCs. Similar studies with neutralizing antibodies to other GC survival factors, such as bFGF [23], also support the concept that endogenous levels of survival factors do not explain the relationship between cell contact and cell survival.

GAP JUNCTIONS OR N-CADHERIN ADHESION JUNCTIONS: WHICH MEDIATE CELL SURVIVAL?

From the previously cited work, it appears that cell contact plays a role in preventing apoptosis. The problem is that GC aggregates are connected by both gap and adhesion-type junctions. Both types of junctions convey information between cells and, therefore, could be involved in the mechanism through which cell contact promotes GC viability. To gain insight into whether either of these junctions play an important role, a co-culture study was conducted. In this study, GCs were cultured with R2C cells, and the R2C–GC complexes were examined for (1) the ability of R2C cells to maintain GC viability, and (2) the type of junctional complexes that were formed between the two cell types. This study revealed that a single contact with an R2C cell was as effective in maintaining GC viability as contact with another GC [20]. However, R2C–GC complexes were not connected by functional gap junctions as assessed by both ultrastructural examination and dye transfer studies [20]. Since R2C cell contact maintains GC viability in the absence of functional gap junctions, it appears that gap junctions are not an essential part of the mechanism by which cell contact prevents GC apoptosis.

If gap junctions are not involved, then adhesion junc-

† *Abbreviations:* GC, granulosa cell; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; $[Ca^{2+}]_i$, intracellular free calcium; IP_3, inositol 1,4,5-triphosphate; PKC, protein kinase C; R2C, non-ovarian cells; HGF, hepatocyte growth factor; and SF, scatter factor.

tions may be the entity that conveys the protective effects of cell contact. An essential component of adhesion-type junctions is N-cadherin, which is expressed by GCs [20] and ovarian surface epithelial cells [23, 30]. Previous work by Farookhi and associates [19, 29] has shown that antibodies to cadherin inhibit GCs from aggregating. Interestingly, about 30–40% of the GCs aggregate in the presence of either the N-cadherin antibody or synthetic N-cadherin peptide [20]. These GCs have a higher incidence of apoptosis than the aggregated GCs of control cultures. This suggests that GCs possess other adhesion molecules, such as other cadherin family members or integrins [31], that allow cells to form cell contacts. However, if the homophilic binding of N-cadherin is disrupted by either the N-cadherin antibody or synthetic N-cadherin peptide, then the anti-apoptotic mechanism associated with cell contact is inactivated. These data strongly support the concept that homophilic binding of N-cadherin on adjacent cells initiates a signal cascade that prevents GC apoptosis.

SIGNAL TRANSDUCTION PATHWAYS REGULATED BY N-CADHERIN AND bFGF

Recent reports have indicated that N-cadherin interacts with the FGF receptor, and this interaction promotes signal transduction through the FGF receptor [32]. In both GCs and ROSE cells, FGF receptors are tyrosine phosphorylated in serum-free medium in the absence of bFGF [23]. This is likely due to the homophilic binding of N-cadherin between adjacent cells, since exposure to an N-cadherin antibody reduces the level of tyrosine-phosphorylated FGF receptor by 50% [23]. Further, FGF receptor antibody studies demonstrate that the FGF receptor is required for cell contact to prevent apoptosis [23]. Taken together, these data indicate that homophilic binding of N-cadherin promotes the tyrosine phosphorylation of the FGF receptor, thereby triggering a signal transduction pathway that prevents apoptosis. In this manner, N-cadherin cell contact is likely to mediate its anti-apoptotic action through the same mechanism that transduces the anti-apoptotic effects of bFGF.

The cellular and molecular mechanism through which N-cadherin-mediated cell contact and/or bFGF inhibits the initiation of apoptosis is largely unknown. Similarly, the cascade of events that induce cells to become apoptotic is poorly defined [33]. It has been proposed that once a cell reaches a certain point in the apoptotic cascade, it is committed to die [34]. For both GCs and ROSE cells, the commitment point appears to be a sustained increase in $[Ca^{2+}]_i$ that occurs within the first few hours of the apoptotic cascade [35, 36]. This is based on the observations that thapsigargin and Bay K, agents that increase $[Ca^{2+}]_i$, induce apoptosis in an RNA/protein synthesis-independent manner [36]. This demonstrates that the components of the apoptotic cascade distal to the increase in $[Ca^{2+}]_i$ are present within these ovarian cells. This is consistent with the concept that calcium-dependent proteases and endonucleases are already present within the cells, and all that is necessary to induce apoptosis is for these enzymes to be activated by an increase in $[Ca^{2+}]_i$ [37, 38]. Since $[Ca^{2+}]_i$ plays an essential role in initiating apoptosis, it is possible that cell contact and bFGF function to maintain calcium homeostasis.

To determine if cell contact is involved in regulating $[Ca^{2+}]_i$ levels, ROSE cells were loaded with the calcium indicator Fluo-3. Fluo-3-loaded ROSE cells were then either co-cultured with ROSE cells or plated on glass Lab-Tek slides. The Fluo-3-loaded ROSE cells that established cell contact with either the ROSE cell monolayer or another Fluo-3-loaded cell fluoresced at relatively low levels (i.e. basal $[Ca^{2+}]_i$ levels). Those Fluo-3-loaded cells that only attached to the glass substrate and did not form a cell contact possessed $[Ca^{2+}]_i$ levels that were three times greater than aggregated cells. Similar experiments were conducted in which Fluo-3-loaded ROSE cells were co-cultured with either N-cadherin-expressing 3T3 cells or parental 3T3 cells (vector control). Those ROSE cells that attached to the N-cadherin-expressing 3T3 cells had a relatively low level of Fluo-3 fluorescence, whereas attachment to the parental 3T3 cells resulted in a 3-fold increase in Fluo-3 fluorescence. Likewise, Fluo-3-loaded ROSE cells that established cell contact in the presence of synthetic N-cadherin peptide had higher $[Ca^{2+}]_i$ levels than controls [36]. Collectively, these studies demonstrate that homophilic N-cadherin binding between adjacent cells maintains calcium homeostasis.

bFGF has been shown to be a survival factor for GCs [23, 39] and ROSE cells [23, 36]. bFGF induces the tyrosine phosphorylation of its own receptor and subsequently enhances PKC activity [40]. While $[Ca^{2+}]_i$ levels are transiently increased in response to bFGF, $[Ca^{2+}]_i$ levels rapidly return to basal levels where they are maintained even in the presence of agents that increase $[Ca^{2+}]_i$ levels [36, 41]. Since PKC activation is involved in stimulating calcium uptake into the cytoplasmic stores [42, 43], it is likely that the bFGF-induced increase in PKC activity accounts for the ability of bFGF to maintain calcium homeostasis and thereby prevent apoptosis. Given that homophilic N-cadherin binding activates (i.e. tyrosine phosphorylates) the FGF receptor, it is likely that the anti-apoptotic effect of N-cadherin-mediated cell contact is also due to an increase in PKC activity.

REGULATION OF $[CA^{2+}]_I$ LEVELS BY SURVIVAL AND APOPTOTIC FACTORS: A WORKING MODEL

Based on the previous discussion, it follows that endocrine, paracrine, or autocrine signals that promote N-cadherin-mediated cell contact will promote cell survival, whereas factors that diminish cell contact will initiate the apoptotic cascade. One likely initiator of ovarian cell apoptosis is HGF, also known as SF. HGF/SF is a pluripotent growth factor that can promote (1) cell dissociation with the loss of

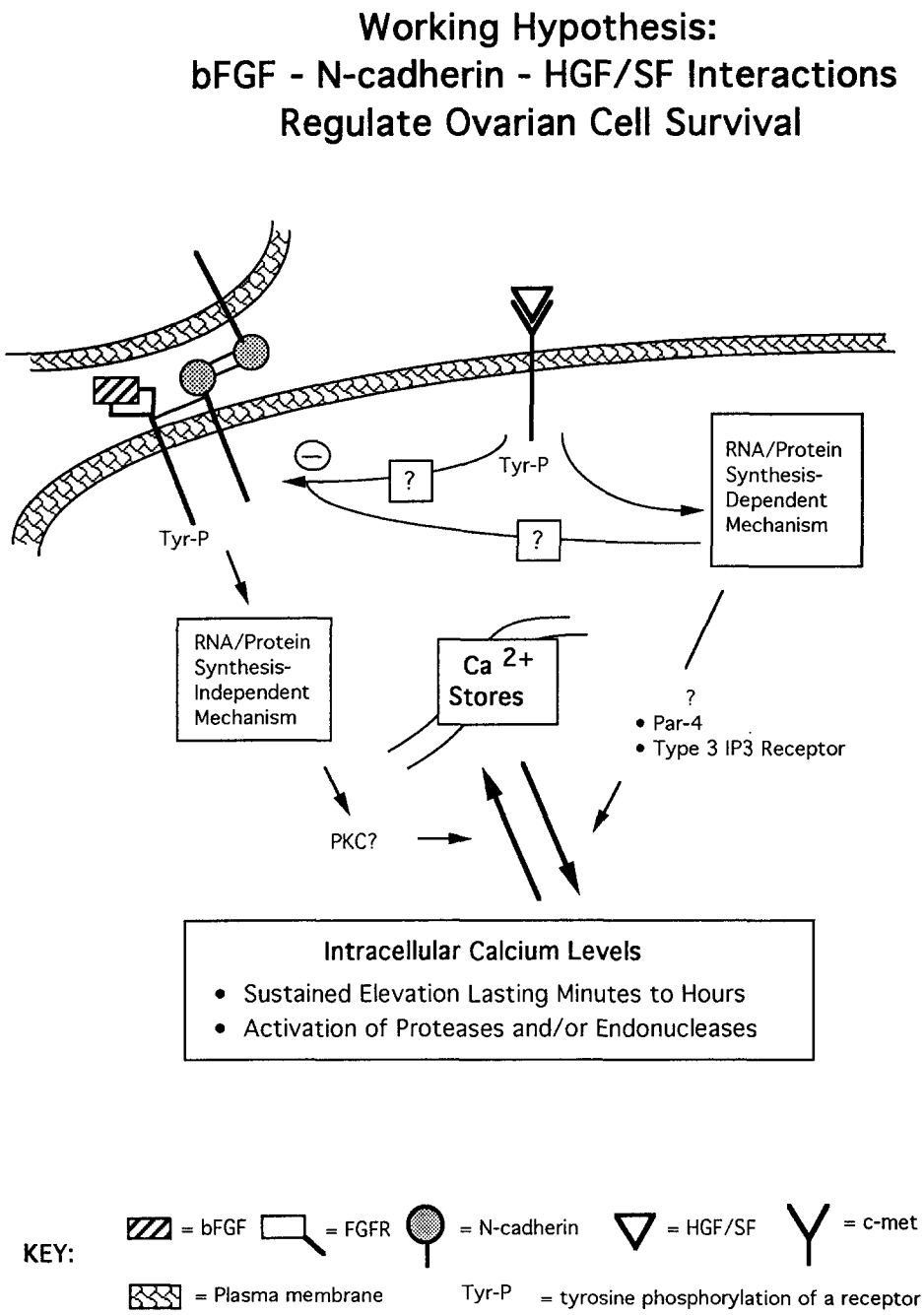

FIG. 1. A working hypothesis that describes the interaction between bFGF, N-cadherin, and HGF/SF in regulating ovarian cell viability. The central component of this hypothesis is that a sustained elevation of $[Ca^{2+}]_i$ levels activates proteases and endonucleases that ultimately induce apoptotic cell death.

adhesion and junctional communication, (2) cell migration, and/or (3) mitosis, depending on the cell type and culture conditions [44, 45]. In ROSE cells, HGF/SF decreases cell contact within 1 hr of treatment, increases $[Ca^{2+}]_i$ levels within 3 hr, and ultimately induces apoptosis [36]. As previously discussed, the ability of HGF/SF to reduce cell contact between ROSE cells could account, in part, for its apoptotic action.

While $[Ca^{2+}]_i$ levels are modulated by various survival and apoptotic factors, a detailed mechanism relating how these different factors interact to regulate $[Ca^{2+}]_i$ levels and thereby control cell viability has not been presented. The following model is proposed as a working hypothesis that describes the interaction between bFGF, N-cadherin, and HGF/SF (Fig. 1). This working model lacks considerable detail, posing more questions than providing answers. Nevertheless, it is presented to identify areas of future study.

The previous data demonstrate that both bFGF- and N-cadherin-mediated cell contact regulate the activation (tyrosine phosphorylation) of the FGF receptor. This, in turn, stimulates a signal transduction pathway that involves the activation of PKC and the maintenance of calcium homeostasis. The isoform(s) of PKC that could account for the anti-apoptotic action of bFGF and N-cadherin is unknown and is currently being studied. It is likely that this survival pathway acts to facilitate the uptake of calcium into the intracellular stores since bFGF prevents an in-

crease in $[Ca^{2+}]_i$ in response to agents that increase $[Ca^{2+}]_i$ levels via very different mechanisms [36]. For example, thapsigargin causes an increase in $[Ca^{2+}]_i$ levels by stimulating the release of calcium from its intracellular stores, while Bay K promotes the opening of calcium channels. Further, the ability of bFGF to maintain calcium homeostasis does not appear to require *de novo* RNA and protein synthesis [36].

The cellular and molecular actions through which HGF/SF induces cell death have not been identified but could include several possibilities. Ovarian surface epithelial cells express c-met, the receptor for HGF/SF [46]. Ligand activation of c-met results in its autotyrosine phosphorylation [44, 45]. Once c-met is tyrosine phosphorylated, a signal transduction pathway is triggered that ultimately results in a decrease in cell contact. In non-ovarian cells, HGF/SF decreases cadherin levels [47] and/or stimulates the tyrosine phosphorylation of catenins [48] with the end-result being a loss of cell contact [49]. Another putative effect of HGF/SF could be to decrease protein tyrosine phosphatase activity, specifically PTP1β. Decreasing the activity of this enzyme would increase the phosphorylation status of catenin, thereby decreasing cell adhesion [50]. Any or all of these actions could be responsible for the ability of HGF/SF to promote disaggregation and, therefore, explain the HGF/SF-induced increase in $[Ca^{2+}]_i$ levels. Finally, it is not known whether HGF/SF-induced cellular disaggregation is dependent on RNA/protein synthesis. If newly synthesized proteins are required, proteolytic enzymes that degrade cadherin and/or catenins would be likely possibilities [37, 38].

In addition to disrupting cell contact, HGF/SF may have additional actions that lead to the deregulation of $[Ca^{2+}]_i$ and ultimately to cell death. In hepatocytes, intracellular levels of IP_3 are elevated within minutes of exposure to HGF/SF and remain elevated for up to 5 hr [51]. IP_3 binds to IP_3 receptors promoting the release of calcium from its stores [43]. This results in an immediate increase in $[Ca^{2+}]_i$, which is generally transient lasting only a few minutes and associated with mitosis [51]. HGF/SF stimulates an increase in $[Ca^{2+}]_i$ in ROSE cells, but HGF/SF-induced changes in $[Ca^{2+}]_i$ differ from those associated with mitosis in that they (1) are delayed, increasing 2- to 3-fold over control values by 3 hr of HGF/SF treatment, (2) are sustained, lasting at least 1 hr, and (3) require *de novo* RNA and protein synthesis [36]. The identity of newly synthesized proteins that may act in concert with IP_3 to increase $[Ca^{2+}]_i$ is unknown and is the subject of current investigations.

Two proteins appear to be likely candidates. First, the expression of an inhibitor of various isoforms of PKC, referred to as Par-4, has been correlated with an inhibition of cell growth and subsequent apoptosis in 3T3 cells [52]. If this protein is expressed during ovarian cell apoptosis, then it is likely to attenuate PKC activity, thereby allowing for a continuous release of calcium from its intracellular stores and thus a sustained elevation in $[Ca^{2+}]_i$ levels. In addition to Par-4, the expression of type 3 IP_3 receptors may be enhanced during ovarian cell apoptosis. The type 3 IP_3 receptor has been shown to be induced during glucocorticoid-stimulated apoptosis of lymphocytes [53]. Activation of the type 3 IP_3 receptor results in a prolonged 4-fold increase in $[Ca^{2+}]_i$ [53]. It is possible then that both Par-4 and type 3 IP_3 receptors are two of several HGF/SF-induced proteins that could result in the deregulation of $[Ca^{2+}]_i$.

FUTURE STUDIES

The present commentary considers the role of N-cadherin-mediated cell contact in regulating the viability of ovarian cells. However, it is becoming increasingly clear that cadherin-mediated cell contact is involved in regulating various cell functions in many different cell types. Examples of the involvement of cadherin include but are not limited to the regulation of cell viability and proliferation of normal intestinal epithelium [54], colon carcinomas [6], and lung carcinomas [55]. In addition, early embryonic development [56] and organogenesis [57, 58] are modulated by cadherin-mediated cell contact. It is in this broader context that future studies are likely to reveal essential physiological pathways that are influenced by cadherin-mediated cell-to-cell contact.

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