Abstract. The hormone 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) regulates a variety of signaling pathways via intracellular Ca$^{2+}$. Modulation of apoptosis is emerging as a promising strategy for treatment and prevention of cancer. Cellular Ca$^{2+}$ has been implicated in triggering of apoptosis, however, the vitamin D/Ca$^{2+}$-dependent targets involved in apoptotic signaling have not been identified. Here, we review our studies on mechanisms of 1,25(OH)$_2$D$_3$-induced Ca$^{2+}$ signaling and Ca$^{2+}$-mediated apoptosis in breast cancer cells. The results obtained demonstrate that 1,25(OH)$_2$D$_3$ regulates Ca$^{2+}$ entry from the extracellular space, Ca$^{2+}$ mobilization from the intracellular stores and intracellular Ca$^{2+}$ buffering. In breast cancer cells, 1,25(OH)$_2$D$_3$ induces the apoptotic Ca$^{2+}$ signal, a sustained increase in concentration of intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_{i}$) reaching elevated, but not cytotoxic levels. This increase in [Ca$^{2+}$]$_{i}$ is associated with activation of Ca$^{2+}$-dependent μ-calpain and Ca$^{2+}$/calpain-dependent caspase-12. Activation of these proteases appears to be sufficient for the execution of apoptosis in cancer cells. Normal mammary epithelial cells resist induction of apoptosis with 1,25(OH)$_2$D$_3$ due to their large Ca$^{2+}$-buffering capacity. The results indicate that the 1,25(OH)$_2$D$_3$-induced cellular Ca$^{2+}$ signal can act as an apoptotic initiator that directly recruits Ca$^{2+}$-dependent apoptotic effectors capable of executing apoptosis. These findings provide a novel rationale for evaluating the role of vitamin D in prevention and treatment of breast cancer.

Apoptosis, Ca$^{2+}$ Signaling and Vitamin D

Apoptosis in cancer. Apoptosis, a highly regulated form of cell death, is the main mechanism for controlling cell number in most tissues (1). Dysregulation of apoptosis underlies in the pathophysiology of proliferative disorders, and a decrease in apoptotic cell death contributes to cancer development and resistance to treatment (2). Moreover, conditions that inhibit or reduce apoptosis are associated with tumorigenesis and increased risk for several types of cancer in humans (1). Understanding how cell fate decisions are made and how cell death pathways are executed or held in check is pivotal to preventing and treating cancer. An induction of apoptosis is considered a potentially effective strategy for cancer treatment (1, 3). The main obstacle for the use of this approach is that the apoptotic molecular targets need to be selectively activated in cancer cells, and those targets need to be conclusively identified for different types of cancer.

Cellular Ca$^{2+}$ and apoptosis. Cellular Ca$^{2+}$ signals have been implicated in triggering and regulating apoptosis, however, Ca$^{2+}$-dependent mediators involved in apoptotic signaling have not yet been identified. This is an important problem because it does not allow a rational search for therapeutic and preventive agents acting via vitamin D- and Ca$^{2+}$-dependent molecular targets in apoptotic pathways. In our laboratory, we investigated mechanisms of 1,25(OH)$_2$D$_3$-induced Ca$^{2+}$ signaling and Ca$^{2+}$-mediated apoptosis in breast cancer cells. The findings, reviewed in this article, demonstrate that 1,25(OH)$_2$D$_3$ induces Ca$^{2+}$-mediated apoptotic death of breast cancer cells and suggest that novel vitamin D analogs that target cellular Ca$^{2+}$ signaling can be exploited in an apoptosis-based approach for prevention and treatment of breast cancer.
bind directly to target proteins. The membrane Ca\(^{2+}\) transport systems control cellular Ca\(^{2+}\) homeostasis. Ca\(^{2+}\) carries information to virtually all processes important for cell life, but it also transmits signals that promote cell death. Spatiotemporal characteristics of the Ca\(^{2+}\) signal determine the type and magnitude of biological responses, e.g. oscillations of cytosolic Ca\(^{2+}\) in pancreatic \(\beta\)-cells underlie the oscillatory pattern of insulin release from these cells (9, 10).

The exact mechanism of Ca\(^{2+}\) signaling in apoptosis is not fully understood. Our group (2, 8, 11-16) and others (4-6) have shown that increases in the concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) occur in the early and late stages of apoptosis. The critical characteristic of the apoptotic Ca\(^{2+}\) signal is a sustained increase in [Ca\(^{2+}\)]\(_{i}\), reaching elevated, but not cytotoxic levels. However, interactions of the cellular Ca\(^{2+}\) signal with molecular Ca\(^{2+}\) targets in cells undergoing apoptosis have not yet been conclusively identified. Ca\(^{2+}\)-dependent intracellular cysteine proteases, the caspases (e.g. caspase-12), and Ca\(^{2+}\)-dependent intracellular neutral proteases, the calpains, are considered as the primary Ca\(^{2+}\)-activated apoptotic targets (2, 13, 17, 18).

Regulation of the Ca\(^{2+}\) signal via intracellular Ca\(^{2+}\) buffers plays a particularly important role in the apoptotic process. A key element of the cytosolic Ca\(^{2+}\) buffering system are the key element of the cytosolic Ca\(^{2+}\) buffering system are the calbindin-D28k dramatically increase the cytosolic Ca\(^{2+}\), buffering capacity and that an increase in Ca\(^{2+}\) buffering via forced expression of calbindin-D\(_{28k}\) protects cancer cells against Ca\(^{2+}\)-mediated apoptosis. It is noteworthy that breast cancer cells do not express endogenous calbindin-D\(_{28k}\) and 1,25(OH)\(_{2}\)D\(_{3}\) does not induce protein expression in these cells (13, 14).

We hypothesized (2, 7, 8) that a sustained increase in [Ca\(^{2+}\)]\(_{i}\) signals the cell to enter an apoptotic state via activation of the Ca\(^{2+}\)-dependent protease \(\mu\)-calpain followed by activation of the Ca\(^{2+}\)/calpain-dependent caspase-12 and downstream caspases (e.g. caspase-3). A lack of expression or low levels of cytosolic Ca\(^{2+}\)-binding proteins (e.g. calbindin-D\(_{28k}\)) may diminish the ability of the cell to buffer [Ca\(^{2+}\)]\(_{i}\) increase and, thus, facilitate induction of apoptosis. On the other hand, agents that induce expression of the intracellular Ca\(^{2+}\) buffers (e.g. calbindin-D\(_{28k}\)) by 1,25(OH)\(_{2}\)D\(_{3}\) in certain normal cell types: intestine, kidney, mammary gland) or suppress pathways for the generation of the apoptotic Ca\(^{2+}\) signal (e.g. Ca\(^{2+}\) channel blockers) may protect against Ca\(^{2+}\)-mediated apoptosis.

1,25(OH)\(_{2}\)D\(_{3}\) and Ca\(^{2+}\)-mediated apoptosis. It is well established that 1,25(OH)\(_{2}\)D\(_{3}\) can induce Ca\(^{2+}\) signals in different cell types. 1,25(OH)\(_{2}\)D\(_{3}\) activates voltage-dependent (VDCC) and voltage-insensitive (VICC) Ca\(^{2+}\) channels and triggers Ca\(^{2+}\) release from the endoplasmic reticulum (ER) through inositol 1,4,5-trisphosphate receptors and ryanodine receptors (2, 7, 8, 13, 14).

1,25(OH)\(_{2}\)D\(_{3}\) generates biological responses via both genomic and nongenomic mechanisms (8, 21-24). Genomic responses utilize signal transduction pathways linked to the nuclear/cytosolic vitamin D receptors (VDRs), while nongenomic, rapid responses utilize signal transduction pathways coupled to the membrane VDRs. Analogs of vitamin D that can act as agonists and antagonists of these pathways have been identified (22, 24, 25). It appears that Ca\(^{2+}\) signals (transient and prolonged) triggered by 1,25(OH)\(_{2}\)D\(_{3}\) can be linked to both membrane and nuclear VDRs (2, 8).

Our group (8, 11, 13, 14) and others (26, 27) have demonstrated that 1,25(OH)\(_{2}\)D\(_{3}\) induces apoptosis in cancer cells and that apoptosis induced by 1,25(OH)\(_{2}\)D\(_{3}\) depends on Ca\(^{2+}\) signaling (2, 10, 13, 28). Nuclear VDRs are believed to determine responsiveness of cancer cells to 1,25(OH)\(_{2}\)D\(_{3}\). However, the efficacy of vitamin D analogs in cancer does not always correlate with their binding affinity to nuclear VDRs, and not all cancer cell lines expressing them respond to 1,25(OH)\(_{2}\)D\(_{3}\). Therefore, signal transduction pathways coupled to both membrane and nuclear VDRs may be involved in regulation of apoptosis (2, 8). Agonists of membrane VDRs can trigger the apoptotic Ca\(^{2+}\) signal and induce cell death without exerting the systemic calcemic activity of 1,25(OH)\(_{2}\)D\(_{3}\) (13).

Below we summarize our findings regarding the role of 1,25(OH)\(_{2}\)D\(_{3}\) in generating Ca\(^{2+}\) signals in breast cancer cells and provide evidence that 1,25(OH)\(_{2}\)D\(_{3}\)-induced Ca\(^{2+}\) signals can determine fate of these cells by apoptosis. These findings may help in the rational search for therapeutic and preventive agents for breast cancer that act via Ca\(^{2+}\)-dependent molecular targets in apoptotic pathways.

1,25(OH)\(_{2}\)D\(_{3}\)-induced Ca\(^{2+}\) Signaling and Apoptosis in Breast Cancer Cells

**Intracellular Ca\(^{2+}\).** 1,25(OH)\(_{2}\)D\(_{3}\) and apoptosis in human breast cancer cells. Our early findings indicate that the plasma membrane VICC and the ER Ca\(^{2+}\) release channels are the main pathways for Ca\(^{2+}\) entry and Ca\(^{2+}\) mobilization in breast cancer cells and that 1,25(OH)\(_{2}\)D\(_{3}\) increases Ca\(^{2+}\) influx through VICC and depletes the ER Ca\(^{2+}\) stores in these cells (10, 14, 15). We suggested that targeting of Ca\(^{2+}\) signaling mediated by VICC and the ER Ca\(^{2+}\) may stand as a novel approach to the treatment and prevention of breast cancer.

We have characterized in detail the regulation of intracellular Ca\(^{2+}\) in the estrogen receptor-positive human breast cell line MCF-7 (10-12). These cells express the highly permeable VICC, but not VDCC. The ER is a major Ca\(^{2+}\) storage compartment, and mobilization of Ca\(^{2+}\) from the ER occurs through inositol 1,4,5-trisphosphate receptor/
Ca\(^{2+}\) release channel, while the ryanodine receptor/Ca\(^{2+}\) release channel is not expressed. 1,25(OH\(_2\))D\(_3\) rapidly increases Ca\(^{2+}\) influx through VICC, depletes the ER Ca\(^{2+}\) stores and elevates basal [Ca\(^{2+}\)]\(_i\). 1,25(OH\(_2\))D\(_3\)-evoked increase in [Ca\(^{2+}\)]\(_i\) is associated with induction of apoptosis in MCF-7 cells (as evaluated by DNA fragmentation and morphological criteria). Treatment of these cells with a Ca\(^{2+}\) ionophore, ionomycin, similarly induces apoptosis. MCF-7 cells loaded with the cytosolic Ca\(^{2+}\) buffer (BAPTA) do not undergo apoptosis in response to 1,25(OH\(_2\))D\(_3\). These findings imply that 1,25(OH\(_2\))D\(_3\) triggers apoptosis in breast cancer cells by causing an increase in Ca\(^{2+}\) entry through VICC and depletion of the ER Ca\(^{2+}\) stores. The resulting elevated [Ca\(^{2+}\)]\(_i\) appears to be sufficient to elicit apoptosis.

Ca\(^{2+}\) and calpain as mediators of apoptosis in breast cancer cells. In a specific study (13), the mechanism of Ca\(^{2+}\)-mediated apoptosis in breast cancer cells was investigated. An increase in [Ca\(^{2+}\)]\(_i\), and depletion of the ER Ca\(^{2+}\) stores with 1,25(OH\(_2\))D\(_3\) induced apoptosis in MCF-7 cells. The increase in [Ca\(^{2+}\)]\(_i\) was associated with activation of the Ca\(^{2+}\)-dependent cysteine protease, μ-calpain. The forced expression of the Ca\(^{2+}\)-binding protein calbindin-D\(_{28k}\) in MCF-7 cells not only attenuated the elevation in [Ca\(^{2+}\)]\(_i\) and μ-calpain activation, but also reduced apoptotic death triggered by 1,25(OH\(_2\))D\(_3\). Similarly, the inhibition of calpain activity by structurally unrelated inhibitors reduced the proportion of apoptotic cells. These results indicate that calpain may play the role of the major protease in apoptotic cell death.

1,25(OH\(_2\))D\(_3\) induces Ca\(^{2+}\)-mediated apoptosis in breast cancer cells, but not normal cells. Another study aimed in comparing the effects of 1,25(OH\(_2\))D\(_3\) on Ca\(^{2+}\) signaling and apoptosis in the cancer and normal human mammary epithelial cells (HMECs) (8). The treatment of MCF-7 breast cancer cells with 1,25(OH\(_2\))D\(_3\) induced a sustained increase in [Ca\(^{2+}\)]\(_i\), and activated Ca\(^{2+}\)-dependent apoptotic proteases, μ-calpain and caspase-12, as evaluated with antibodies to active (cleaved) forms of the enzymes and the calpain peptide substrate. The selective inhibition of the Ca\(^{2+}\)-binding sites of μ-calpain reduced apoptotic indices in 1,25(OH\(_2\))D\(_3\)-treated cells. 1,25(OH\(_2\))D\(_3\) did not induce apoptosis in normal HMECs, as evaluated by DNA fragmentation, loss of plasma membrane asymmetry and morphological criteria. In HMECs, 1,25(OH\(_2\))D\(_3\) triggered a transient Ca\(^{2+}\) response, which was not accompanied by calpain or caspase activation. HMECs, but not MCF-7 cells, expressed the Ca\(^{2+}\)-binding protein calbindin-D\(_{28k}\) and were capable of buffering the apoptotic (i.e. non-cytotoxic) [Ca\(^{2+}\)]\(_i\) increases induced by the Ca\(^{2+}\) ionophore ionomycin.

These results support the hypothesis that the Ca\(^{2+}\) entry, mobilization, and buffering mechanisms differ dramatically in breast cancer cells and normal mammary epithelial cells. Ca\(^{2+}\) handling by normal HMECs (Ca\(^{2+}\) entry and Ca\(^{2+}\) mobilization pathways allowing only a transient 1,25(OH\(_2\))D\(_3\)-induced Ca\(^{2+}\) increase and a low Ca\(^{2+}\)_buffering capacity) seem sufficient to protect those cells from Ca\(^{2+}\)-mediated apoptosis. Ca\(^{2+}\) handling by breast cancer cells (Ca\(^{2+}\) entry and Ca\(^{2+}\) mobilization pathways permitting generation of a sustained, prolonged increase of [Ca\(^{2+}\)]\(_i\) and a low Ca\(^{2+}\)-buffering capacity) allows the induction of apoptosis with 1,25(OH\(_2\))D\(_3\) in these cells. The findings clearly imply that differences of Ca\(^{2+}\) regulatory mechanisms in breast cancer cells vs. normal mammary epithelial cells underlie resistance of normal cells and susceptibility of cancer cells to 1,25(OH\(_2\))D\(_3\)-induced, Ca\(^{2+}\)-mediated apoptosis.

Conclusion

The series of studies reviewed here has identified the novel apoptotic pathway regulated by 1,25(OH\(_2\))D\(_3\): increase in [Ca\(^{2+}\)]\(_i\), → μ-calpain activation → caspase-12 activation → apoptosis. These investigations indicate that the 1,25(OH\(_2\))D\(_3\)-activated apoptotic molecular targets are Ca\(^{2+}\)-dependent calpain and Ca\(^{2+}\)/calpain-dependent caspase-12. Importantly, calpain and caspase-12 are activated by other Ca\(^{2+}\)-regulatory compounds as well (29-32). The Ca\(^{2+}\)-mediated apoptotic mechanism cannot be activated by 1,25(OH\(_2\))D\(_3\) in normal mammary epithelial cells because they are protected from Ca\(^{2+}\)-mediated apoptosis via adequate buffering of [Ca\(^{2+}\)]\(_i\) increase and limited permeability of the VICC. The differences in Ca\(^{2+}\) signaling between breast cancer and normal cells can be exploited to rationalize the further search for selective anticancer vitamin D analogs effective in treatment of tumors susceptible to induction of Ca\(^{2+}\)-mediated apoptosis.

Taken together, the findings reviewed demonstrate that Ca\(^{2+}\)-mediated apoptosis appears to be an inducible mechanism for cell death in breast cancer. Clearly, cellular Ca\(^{2+}\) can act as an apoptotic initiator and directly recruit Ca\(^{2+}\)-dependent apoptotic effectors capable of executing apoptosis in breast cancer cells. Research of 1,25(OH\(_2\))D\(_3\)-regulated Ca\(^{2+}\) signaling pathways will allow the development of new chemotherapeutic and chemopreventive vitamin D analogs for modulation of apoptosis in cancer, which target the cellular Ca\(^{2+}\) entry and Ca\(^{2+}\) mobilization pathways, intracellular Ca\(^{2+}\) buffers, and Ca\(^{2+}\)-dependent apoptotic proteases. Further research is necessary to identify molecular targets involved in 1,25(OH\(_2\))D\(_3\)/Ca\(^{2+}\)-mediated apoptosis, including complimentary studies using preclinical animal models. The studies reviewed here highlight the need to further define 1,25(OH\(_2\))D\(_3\)-mediated Ca\(^{2+}\) signaling at the cellular and molecular level in relation to prevention and treatment of cancer.
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