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In situ clinical evidence that zinc levels are decreased in breast invasive ductal carcinoma

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Abstract

Purpose—Altered zinc levels in malignant cells versus their normal cells have important implications in the development and progression of several cancers. Prostate, pancreatic, and hepatocellular carcinomas exhibit consistent marked zinc decrease in situ in the malignant cells, and other cancers (such as kidney, lung, and thyroid) also exhibit decreased tissue zinc levels. However, zinc levels are increased in breast cancer tissue compared to breast normal tissue, and the contemporary dominant view is that zinc is increased in invasive ductal carcinoma. This has important implications regarding the role and effects of zinc in breast malignancy compared to other cancers, which caused us to initiate this study to either confirm or challenge the contemporary view of an increased zinc level in the invasive ductal malignant cells.

Methods—We employed dithizone staining of breast tissue sections and tissue cores to determine the relative in situ cellular zinc levels specifically in the invasive ductal malignant cells as compared to normal ductal epithelium. This approach had not been employed in any of the reported breast studies.

Results—The results revealed that the zinc levels are consistently and markedly decreased in the ductal malignant cells as compared with higher prominent zinc levels in the normal ductal epithelium. Decreased zinc is evident in Grade 1 well-differentiated malignancy and in Grade 2 and Grade 3 carcinomas. Among the twenty-five cancer cases in this study, none exhibited increased zinc in the invasive ductal carcinoma compared to the zinc level in the normal ductal epithelium.

Conclusions—The decreased zinc levels in breast invasive ductal carcinoma is consistent with prostate, pancreatic, and liver carcinomas in which the decrease in zinc is a required event in the development of malignancy to prevent cytotoxicity that would result from the higher zinc levels in the normal cells. This new understanding requires a redirection in elucidating the mechanisms and factors regarding the regulation of zinc in breast cancer, its potential translational applications as possible biomarkers, and for treatment of breast invasive ductal carcinoma.

Keywords

Breast cancer; Invasive ductal carcinoma; Zinc; In situ tissue analysis; Prostate cancer; Pancreatic ductal carcinoma; Hepatocellular carcinoma; Zinc cytotoxicity and carcinogenesis

Introduction

About 230,000 new cases of invasive breast cancer and 40,000 deaths occur yearly among US women, and added to this are approximately 65,000 new cases of in situ breast cancer [1]. Thus, breast cancer is the second most common cancer (after skin cancer) and the second leading cause of cancer death (after lung cancer) in women. Such statistics dictate the necessity for the identification of the factors and events associated with the development and progression of breast cancer malignancy, which could lead to effective biomarkers for early breast cancer and to efficacious treatment and perhaps prevention of breast cancer.

Altered zinc concentrations have been identified in association with the development of malignancy in several cancers [2, 3]. In most of these cancers, the concentration of zinc in the malignant tissue has been reported to be decreased when compared to the zinc levels in corresponding normal or benign tissue. The most notable exception is breast cancer (invasive ductal carcinoma) in which the zinc level is significantly greater than the zinc level in normal/benign breast tissue. This relationship is based on several corroborating reports (such as [4–10]). Most importantly, the increased zinc level in the breast cancer tissue has been interpreted to reflect an increase in zinc in the malignant ductal cells as compared to the zinc level in normal ductal epithelium, thereby demonstrating that increased cellular zinc promotes the development and progression of malignancy. This is the current prevailing view of the status of zinc in breast cancer.

Because of the important implications of zinc in the development and progression of malignancy in specific cancers, it is extremely important that the status and role of zinc in breast cancer and in all cancers be correctly and definitively established. Achieving this requires that the relative zinc levels must be established by appropriate in situ determination of zinc levels directly and specifically in the malignant cells and the corresponding normal cells in human tissue sections, as has been done for prostate, liver, and pancreatic carcinomas [11–15]. In these cancers, the in situ zinc staining has demonstrated the marked and consistent decrease in the malignant cells as compared to the corresponding normal cells, which is consistent with the corresponding decreased zinc that is identified in the tissue preparations.

This approach had not been employed in any of the reported breast cancer studies. Instead, the zinc levels were determined with tissue extracts and/or tissue analyses of cancer tissue “areas” versus normal/benign tissue “areas.” More than other cancers, the histological heterogeneity and composition of breast, including major tissues such as adipose and muscle along with the ductal structures, present a challenging difficulty in relating the tissue zinc levels specifically to the malignant cells versus normal epithelium.

Therefore, we initiated a study in which we employed the in situ zinc staining method in tissue sections to determine the relative zinc levels in the malignant cells versus the normal ductal epithelium in breast tissue sections. We now present clinical evidence that the zinc levels in breast tissues are markedly and consistently decreased in the invasive ductal malignant cells as compared to the higher zinc levels in the normal ductal epithelium. This provides a new understanding of the implications of zinc in breast invasive ductal carcinoma, which requires a new direction in elucidating the mechanisms and factors regarding the regulation of zinc in breast cancer, and its potential translational applications as possible biomarker, and for treatment and possibly prevention of breast cancer.

Methods

Breast tissue microarray slides (TMAs) were obtained from US Biomax, Inc. Breast tissue sections prepared from archived breast tissues were provided from the University of Maryland Greenebaum Cancer Center Pathology Biorepository and Research Shared Service. Deparaffinization was achieved by placing the slides in an incubator at 60 °C for up to 1 h, followed by immersion of the slides in 95 % ethyl alcohol, followed by rinsing with triple-distilled H₂O. The slides containing tissue sections or TMA cores were immersed in freshly prepared dithizone solution [16] containing the addition of pyridine at five times the concentration of dithizone, which stabilizes the ZnDithizone chromogen [17]. The slides were immersed in the dithizone solution generally for up to 30 min until optimal zinc staining was achieved. The slides were rinsed with triple-distilled water, a drop of glycerol was pipetted over the tissue section/cores, and a coverslip was applied.

The stained slides were examined by light microscopy using a multi-headed Reichert Omega Model #4000 microscopy system equipped with a high-definition, two-megapixel camera and a desktop computer for capturing and storing digital images. On occasion, H&E staining was performed for histopathological verification of the cells/tissues attained with dithizone.

We employed dithizone staining for this study because of its strong zinc-binding formation constant of $\log K_f \sim 15$, which will bind essentially all of the cellular zinc [18]. The visualized stain intensity (light microscopy black staining) of the ZnDithizone chelate provides a measurement of the relative total zinc in the cells. This determination of the total zinc levels was advisable in order to relate the in situ zinc measurements to the other reported studies in which methods that determined total zinc in the breast tissues were employed.

The reference to the grades follows the US Biomax information provided with the malignant cores representing the histopathology of the respective cancer cases. Grade 1 = well-differentiated malignancy; Grade 2 = moderately differentiated malignancy; Grade 3 = poorly differentiated malignancy.

Results

Figure 1 shows representative results of dithizone staining of a TMA slide containing cores of Grade 1, Grade 2, Grade 3 invasive ductal carcinomas and cores of normal breast tissue. The normal ducts exhibit prominent ZnDithizone staining of the ductal epithelium. In

contrast, the invasive malignant ducts show minimally detectable zinc staining, whereas surrounding and adjacent stromal tissues show some zinc staining. Notably, the decrease in zinc is evident in Grade 1 well-differentiated malignancy and in moderately differentiated Grade 2 malignancy, and in Grade 3 poorly differentiated malignancy.

To confirm and extend the results obtained in Fig. 1, we obtained and stained additional TMA slides. Figure 2 is taken from another TMA slide containing Grade 2, Grade 3, and normal cores. The results again reveal the prominent zinc staining in the normal ductal epithelium, whereas Grade 2 and Grade 3 malignancies exhibited minimal detection of zinc, although adjacent stromal tissues exhibit prominent zinc staining.

In order to corroborate and extend the results obtained with the TMA cores, we obtained archived tissue sections of two confirmed invasive ductal carcinoma cases, which included cancer-adjacent normal and invasive ductal carcinoma sections from each case. Figure 3 shows the marked decreased zinc in invasive ductal carcinoma cells as compared to the dominant zinc staining in the normal ductal epithelium. Consequently, these results confirm and extend the decreased zinc observed in the cases represented in the TMA cores.

This study included cancer tissues from twenty-five cancer cases and normal tissues from twelve cases that included either cancer-adjacent normal tissue or normal tissue from non-cancer subjects. Among these samples, there was no case in which the invasive ductal carcinoma exhibited an increased zinc level compared to the normal ductal epithelium.

Discussion

Over the past several decades, there has been much interest in the status and relationship of zinc in cancers. As such, many reports have presented the concentrations of zinc in cancer versus normal/benign tissues, from which there have been varying results among the different cancers, and even within the same cancer. Some of this variability is due partly to the different conditions employed relative to the type of tissue preparations being analyzed, the method and technique of the zinc assays, and other such conditions. Other variables are associated with the subject populations involved in the studies, such as stage of cancer and existing confounding conditions. Consequently, for many cancers, the existing information is insufficient to reach a conclusive establishment of the zinc status.

However, it is well established that tissue zinc levels are decreased in prostate, liver, and pancreatic carcinomas, and supporting evidence also for thyroid, lung, gall bladder, and kidney cancers [3, 12–15, 19]. In contrast, several consistent reports [e.g., 4–10, 19] established that tissue zinc levels are increased in breast cancer, which has led to the contemporary understanding that zinc is increased in invasive ductal carcinoma. As such, the status of zinc in breast cancer would be different from the decreased zinc that exists in other cancers and most notably in prostate, pancreatic, and hepatocellular carcinomas [11–15]. We had established that those cancers exhibit the decrease in zinc in Grade 1 well-differentiated malignancy, and even in the premalignant lesions (prostate intraepithelial neoplasia; pancreatic intraepithelial neoplasia). The decrease in zinc is an early event in the development of malignancy in those cancers, and it persists in advancing Grade 2 and Grade

3 malignancies. In addition, the decrease in zinc exists in all or most cases of those respective carcinomas, to the extent that cancer cases in which the malignancy retains the higher zinc levels found in the corresponding normal cells rarely, if ever, exist. The decrease in zinc in those cancers is essential to prevent malignant cell cytotoxicity that will result from the higher zinc levels that exist in the normal cells. Consequently, those cancers evolved with mechanisms that prevent the accumulation of the higher cytotoxic zinc levels in the malignant cells, and that maintains a lower cellular zinc level that is optimal for the growth, proliferation, metabolism, and malignant activities of the malignant cells.

With this understanding, a status of increased zinc in the malignant cells in situ in breast cancer would indicate that increased zinc facilitates malignancy, including its promotion of proliferation and its inhibition of apoptosis. Such effects are opposite to the well-established cytotoxic effects of zinc, which include its inhibition of proliferation and its induction of apoptosis [3, 20]. If so, this would be an intriguing and very relevant relationship as to how zinc exhibits such opposite effects in breast malignancy compared to other cancers. Alternatively, we considered that the reported increase in zinc in breast cancer tissues might not reflect the status of zinc specifically in the ductal malignant cells as compared to the normal ductal epithelium. The absence of any reported studies that employed the direct in situ determination of zinc levels specifically in ductal malignant cells and in normal ductal epithelium dictated that it was most important to establish the correct relationship.

The above considerations caused us to initiate the studies presented in this report. The results of this study reveal that the status of decreased zinc in breast invasive ductal carcinoma is consistent with prostate, pancreatic, and liver carcinomas as represented in Fig. 4. It is also notable that the decrease in zinc in breast invasive ductal carcinoma is evident in Grade 1 well-differentiated malignancy and is seen in Grade 2 moderately differentiated malignancy and Grade 3 poorly differentiated malignancy. This is identical to prostate, liver, and pancreatic carcinomas [12–15]. As such, it is highly likely that the decrease in zinc is an essential early event in the oncogenic development of breast malignancy, so as to prevent the cytotoxic effects of zinc accumulation in the malignant cells, as in the other cancers.

We must emphasize that the results of this study do not contradict or cast doubt on the reports that demonstrated increased zinc in breast cancer tissues compared to breast non-cancer tissues. The issue is the interpretation that the decreased tissue zinc levels represent a decrease specifically in the invasive ductal malignant cells compared to the normal ductal epithelium. The cases included in this study did not reveal an increase in zinc in the ductal malignant cells compared to the normal ductal epithelium. However, the in situ staining has provided a visualization of the diversity of zinc staining in the breast tissue components that are adjacent to the ducts, such as adipose tissue, connective tissue, smooth muscle. Figure 5 exemplifies the impact of such confounding conditions. The TMA slides (Fig. 5A) show the diverse staining that we commonly observed in the normal and cancer cores. Especially relevant is the low staining of the malignant ducts (small clear areas) compared to the heavily stained “stromal tissue” areas. When one considers that the diameter of the TMA cores approximates 1500u, the localized area of malignant ducts without immediate adjacent “stromal tissue” might be a diameter of ~50u–100u. Consequently, it is most likely that the methods employed in the reported studies would not define or distinguish the difference in

zinc levels specifically in the malignant ducts versus normal ductal epithelium. Figure 5B is a tissue section that reveals the extremely low zinc staining in neighboring adipocytes which would have the effect of decreasing the zinc level assigned to the normal ductal epithelium. Figure 5C reveals the high zinc staining of necrotic tissue, which can be adjacent to the malignant ducts, thereby increasing the zinc level assigned to the malignant ducts. Such confounding conditions make it evident that the use of macroscopic tissue extracts for determining the zinc status specifically in the malignant cells versus the normal ductal epithelium malignant ducts is not a reliable approach. Also micro-methods (such as XRF) that detect zinc in tissue sections must also have a microscopic target that ensures detection of the zinc levels specifically in the malignant cells and in the normal ductal epithelium. We believe that the visualized in situ tissue zinc staining in the cells provides the most reliable method for specifically establishing the cellular level of zinc in the malignant cells versus normal ductal epithelium.

It is notable that Garg et al. [21] report that Zn, Cu, Mn, Co, Se, Br, As, Sb, Cd, Na, K, P, Cl, and Mg are elevated in cancerous tissue, whereas lower levels are observed for Fe, Cs, I, and Sr. Ng et al. [4] reported that Al, Br, Ca, Cl, Co, Cs, Fe, K, Mn, Na, Rb, and Zn are significantly higher in the cancer tissues. Majewska et al. [22] reported that P, S, K, Ca, Mn, Fe, Se, Rb, Ni, Zn, and Pb are increased in breast cancer tissue. Such results raise the question as to why these simultaneous changes in all of these elements should be expected to exist in the malignant cells, and as relevant factors being involved in the transformation of the normal cells to malignant cells. Seemingly, such results should have revealed this conundrum, and required verification of the direct association of such widespread element changes being associated with the malignant cells.

It is also relevant that reports [23–25] have demonstrated that exposure of MCF-7 (malignant breast cell line) to zinc-supplemented medium results in inhibition of proliferation and increased apoptosis. Especially relevant is the cytotoxic effect of ZnCl₂ on MCF-7 cells [24], which is identical to the inhibition of cell proliferation that we have reported for prostate cells [26]. Reports, such as [27], purport to show that increased zinc prevents cytotoxic effects. However, this effect is demonstrated in “zinc-depleted dying” cells, i.e., an experimental unphysiological condition.

Such relationships are consistent with the clinical evidence presented in this report that, in breast cancer, zinc is decreased in the malignant cells as compared to the normal/ benign cells. At the very least, this raises question as to the validity of the contemporary conclusion that the reported increased zinc concentration in breast cancer tissue compared to normal/ benign tissue reflects the status of zinc in the malignant versus normal/benign cells. The latter would translate to the concept that the development and/or progression of breast cancer involves a transformation in which zinc is increased in the malignant cells, which has and will continue to drive the direction of breast cancer research relative to the implications of zinc. This present study translates to the opposite concept, i.e., that a decreased zinc accumulation occurs in the development and/or progression of the malignant cells in breast cancer.

It will be important to expand our studies to include a larger population of breast cancer cases in order to corroborate and establish further the consistency of the decreased zinc in association specifically with invasive ductal malignancy. We expect that most (if not all) cases of invasive ductal carcinoma will exhibit this relationship. If so, its implications as a required event for the oncogenic development of breast malignancy should be the focus and direction of future research in breast cancer. It is important to determine the factors and mechanisms associated with the decrease in zinc the development of invasive ductal carcinoma, and its potential application for the identification of early biomarkers, and for an efficacious treatment and perhaps prevention of breast cancer.

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Fig. 1.

Representative in situ ZnDithizone staining of a TMA slide containing cores of Grade 1, Grade 2, Grade 3 invasive ductal carcinomas and normal breast. **A** The *yellow circles* are areas of normal ducts, which are shown in the *enlarged figures*. **B** The *red circles* are areas of invasive ductal carcinoma which are shown in the *enlarged figures*. The normal ductal epithelium exhibits prominent ZnDithizone *black* staining. The invasive ductal carcinoma areas exhibit minimal detection of ZnDithizone staining, whereas adjacent stromal tissue exhibits ZnDithizone staining. Original magnifications = 40×;100×. (Color figure online)

Fig. 2.

Representative in situ ZnDithizone staining of a TMA slide containing cores of Grade 2, Grade 3 invasive ductal carcinomas and normal breast. The *yellow circles* are areas that are enlarged in the second row. The normal ductal epithelium exhibits prominent ZnDithizone *black staining*. The invasive ductal cells exhibit minimal ZnDithizone staining although ZnDithizone staining is evident in adjacent stromal tissue. (Color figure online)

Fig. 3.

In situ ZnDithizone staining of tissue sections from two cases (**A, B**) of invasive ductal carcinoma. The cancer-adjacent normal ducts exhibit prominent ZnDithizone staining, and the invasive malignant ducts exhibit decreased zinc staining. **C** H&E of tissue sections of **B**

Fig. 4.
Similar decreased zinc in malignant cells compared to their corresponding normal cells in breast, prostate, pancreatic, and hepatocellular carcinomas as revealed by in situ ZnDithizone staining

Fig. 5.

Representations of the confounding effects of zinc levels of diverse tissues on the determination of the zinc levels in normal ducts and the malignant ducts in invasive ductal carcinoma. **A** Diverse ZnDithizone staining that accompany tissue cores of normal areas of ductal epithelium and areas of invasive ductal carcinoma (*yellow areas*). **B** Beast tissue section showing the lack of ZnDithizone staining in adipose tissue adjacent to ZnDithizone-stained normal ductal area. **C** Tissue section of invasive ductal carcinoma showing the high ZnDithizone staining of necrotic tissue adjacent to the area of invasive malignant cells that does not exhibit ZnDithizone staining. (Color figure online)