Ultrastructural Features of Invasion in Chick Embryo Liver Metastasis of Yoshida Ascites Hepatoma

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SUMMARY

Establishment of metastatic growth by Yoshida Ascites Hepatoma 7974 in the chick embryo liver has been studied with light and electron microscopy. Tumor cells lie as emboli within the sinusoids for the first 24 hr and then begin to divide and invade the parenchyma by 48 hr after inoculation. At this time, tumor cells are seen to extend pseudopodia beneath the endothelium, which in many instances is disrupted by the advancing tumor. There is a concurrent loss of tight junctions and epithelial organization of the tumor cell islands. By 72 hr after inoculation, tumor cells are well established in the parenchyma, in intimate contact with hepatic cells. The parenchymal cells, however, show no obvious ill effects of this host-parasite interaction.

INTRODUCTION

Cancer is a lethal disease because it disseminates. While the establishment of metastasis is the focus of therapeutic attention, it is as yet poorly understood. One model that provides considerable insight into the metastatic process is the vascular dissemination of cancer cells in the chick embryo. Earlier studies from this laboratory have described the establishment of a number of tumors in the chick embryo (10–12). We have also characterized the morphology of Yoshida Ascites Hepatoma 7974 both in the rat peritoneum (14) and in initial phases of metastasis in the chick embryo liver (15). This paper, which represents a continuation of studies with this tumor, discusses the specific cellular interactions involved in the establishment of liver metastases in the chick embryo as seen at the electron microscopic level.

The chick embryo-host model system provides a number of useful advantages: (a) the ability to grow and compare a variety of tumors regardless of the original host, because the chick embryo is incapable of mobilizing an effective immunological response; (b) the ability to sample large numbers of metastases at extremely early stages; (c) the earlier experimental observations starting with Bender et al. (2), showing that from a histopathological point of view the metastatic process in the chick embryo bears close relationship to similar metastatic processes in a variety of mammalian systems (7, 8, 10–12).

MATERIALS AND METHODS

Techniques used in these studies have been described in our earlier reports (10, 15). Briefly, 0.025 ml 7-day-old Yoshida Ascites Hepatoma 7974, with 10% packed cell volume, was injected into the vitelline vein of 10-day-old chick embryos. Samples of the inoculum were fixed, and chick embryo livers were examined 24, 48, and 72 hr after inoculation. Some changes seen in tumor cell organelles in these embryos have been reported previously (15). Controls were inoculated with cell-free supernatant from the ascites fluid and the livers were examined at the same time intervals. Light microscopic material was fixed in Stieve's mixture. Electron microscopic material was fixed in a sucrose-containing Veronal acetate-buffered 2% OsO₄ mixture and embedded in Epon-Araldite. Sections were examined with a Philips 100B electron microscope.

RESULTS

Light Microscopy. The donor ascites tumor (Fig. 1) consisted predominantly of balls of cells with a distinct epithelial appearance and with no observable mitoses as described previously (14). Mitotic rate is a function of age in ascites tumor. AH 7974, younger than 7 days, does show some mitosis, and therefore only “plateau-phase” tumor was used. After 24 hr, numerous tumor emboli could be found in the liver sinusoids (Fig. 2a). While these emboli seemed to have some changes in shape, the cells and balls of cells were the same size as those of the donor inoculum. They were clearly in the sinusoids, not extravascular, and the mitotic rate was still negligible.

At 48 hr (Fig. 2b), several changes were obvious. The tumor foci had enlarged considerably and their outlines were no longer clearly demarcated, so that it was impossible to tell whether the tumor cells were still within sinusoids, while the...
number of foci remained essentially the same. The number of tumor cells had nearly doubled within the space of 24 hr, and they were still actively proliferating as evidenced by the large number of observable mitoses. From 3 to 5% of all visible tumor cells were in mitosis.

By 72 hr (Fig. 2c), the growth was even more dramatic. The tumor cell foci had again at least doubled in size and now occupied a considerable portion of the total cross-sectional area of the liver. The mitotic rate was still high. By this stage the definitive architectures of the tumor had become discernible. The main pattern was one of fairly well-defined masses of tumor cells, considerably larger than the ascites islands. These masses appeared to be predominantly intrasinusoidal, but this was difficult to assess. A 2nd distinct pattern consisted of tumor-lined cysts containing free-floating tumor and blood cells (Fig. 2c). These were unusual at this stage but quite common at 7 days, when they had become much fuller and larger. Fig. 2c also illustrates an intermediate architecture which consisted of less compact masses of tumor cells much like those already described, but showing clear separations between individual cells.

Epoxy embedment and toluidine blue staining further clarified the formation of the tumor-lined cysts. Initiation of cyst formation could be seen as early as 48 hr (Fig. 3). In certain areas elongated tumor cells could be seen lining compartments within the sinusoids. These compartments contained actively proliferating tumor cells. They remained separate from the liver parenchyma and seemed to lead to loose intrasinusoidal growth. With or without cyst formation, much of the tumor growth by 72 hr was still intrasinusoidal.

The majority of tumor foci, however, showed at the periphery active invasion and separation of liver parenchymal cells from one another by 72 hr (Fig. 4). Many tumor cells had been completely incorporated into the structure of the liver parenchyma. These tumor cells seemed to show a tighter association with 1 another than did adjacent tumor cells that remained intrasinusoidal. The predominant architecture of the tumor consisted of loose single cells. However, at sites of interaction with liver parenchyma the tumor cells were tightly associated with one another.

Electron Microscopy. The ultrastructure of the donor ascites tumor has been extensively discussed (9, 14, 21) and will only be summarized here. The tumor grows as balls of cells in the peritoneum. Each cell within such islets has 2 specific kinds of surfaces (Fig. 5): a highly interdigitated intercellular border and a free surface with numerous small microvilli. There is a tight junction at every tumor-fluid interface (Fig. 6). Although other types of junctional complexes are scattered, no exceptions have been observed to this rule of terminal tight junctions.

At 24 hr after i.v. inoculation, emboli were easily found in the liver sinusoids. The cells within these emboli showed many morphological changes, which have been discussed in an earlier paper (15). Frequently, the shape of these emboli was considerably different from the ascites tumor islets, but there were still tight junctions at every cell-fluid boundary (Fig. 7), suggesting that the change in shape was a result of mechanical deformation due to the size of the vascular channels. The tumor cells were often in contact with sinusoidal lining cells such as Kupffer cells (Fig. 8). There was no apparent interaction and the contact seemed to be only a fortuitous juxtaposition. The term “interaction” is used to imply an active process, as contrasted with passive contact. The specific meaning of the term “interaction” is best characterized in each case by the accompanying figures.

The 48-hr picture was strikingly different. Mitoses were now present and the organized islet architecture was disappearing. The cell islands seemed to be falling apart. Tight junctions were now frequently missing and the tumor cell masses had irregular outlines due to amoeboid movement of the cells (Fig. 10). The previously simple cell surfaces had become far more complex. One new type of cell process consisted of tortuous pseudopods (Figs. 10 and 11) showing a very involved series of close contacts with the sinusoidal lining cells and even with the hepatocytes underneath. Some tumor cells also showed an increase in number, length, and complexity of microvilli, particularly in regions next to hepatocytes (Fig. 9). Much more massive amoeboid pseudopods, with predominantly organelle-free cytoplasm, were also common, and such pseudopods could even be seen deeply invaginated within the underlying liver parenchyma (Figs. 12 and 13).

These cell processes probably all are used by the tumor cells as invasive devices. In order to understand invasion in chick embryonic liver, however, the structural organization of the liver sinusoids and parenchyma should first be discussed. Fig. 15 is a low-power view of a normal 14-day-old sinusoid and the surrounding parenchyma. The sinusoidal lining consists of a variable number of layers and cell types (1). There are frequent discontinuities in the lining, so that small channels connect the Disse spaces to the sinusoidal space. In general, the layer next to the vascular channel is extremely thin endothelium, which may in many places be the only lining layer. Phagocytic Kupffer cells are frequent and lie on top of the endothelium, i.e., on the luminal side. They may send out plates of cytoplasm over the endothelium for a considerable distance. Fibroblasts (6) are found under the endothelium and these are also thin, with long plate-like processes. The fibroblasts are sparsely distributed and absent from most planes of section. When present, they are found mostly in the “corner” formed by the anastomosis of tubes of liver parenchyma. Finally, there is a thin layer of unevenly distributed collagen fibrils. Thus, the sinusoidal lining consists of 4 overlapping layers: Kupffer cell, endothelium, fibroblasts, and collagen fibrils. However, all of these layers are discontinuous and any 1 of them may be the outermost layer in a particular place. The leaflets of each of these cells look similar, and it is usually difficult to distinguish which leaflets belong to which cell type. There was no observed change in the organization, distribution, or number of discontinuities of these cells from 12 to 14 days in the embryogenesis of the chick liver. These discontinuities did not appear to represent artifacts introduced during the fixation and embedding process.
By 72 hr after i.v. inoculation, tumor cells were found under the endothelium and in intimate association with liver parenchymal cells at the boundaries of the tumor focus. The center of the focus now showed a distinct architecture which was quite different from that of the ascites tumor. There was variability in tightness of packing, but there were no tight junctions between tumor cells, although there were occasional desmosomes. Fragments of sinusoidal lining cells appeared throughout the matrix of the tumor (Fig. 14). This seemed to be the only stroma of the tumor, since no collagen fibers were seen. At this time single tumor cells were found within sinusoids in uninvolved areas of liver. There, tumor cells may reflect embolic extension from large masses of tumor cells, either hepatic or extrahepatic, or the active migration of tumor cells.

Although the events at 2 days seemed to include disruption of the wall of the sinusoid, at 3 days many sinusoids with intact linings were surrounded by tumor cells (Fig. 16). These surrounded vessels showed more and larger fibroblasts than normal, indicating a hyperplastic and hypertropic response to the tumor cells. At 72 hr, tumor-parenchymal interactions were frequent. The usual observation was simply of tumor cells having long adjacent borders with normal-looking liver cells.

Two kinds of interaction other than contact and the obvious dissociation of liver cells (Fig. 17) from one another were occasionally seen. One such interaction was a thickening in the tumor cell membrane with a corresponding thickening of the adjacent liver cell membrane (Fig. 18). Perhaps this suggests the presence of some sort of rudimentary junctional complex, although this is conjecture. In addition, liver cells frequently protruded into invaginations of tumor cell membranes. This was observed in about 0.1 of the cell contacts photographed. At the tip of these invaginations, small pinocytic vesicles were seen (Fig. 19). This is a peculiar arrangement of cells and pinocytosis. The possibility that the tumor cell absorbs liver cell material in this manner is worth considering. Even here, there was no evidence of liver cell necrosis.

**DISCUSSION**

In contrast to lymphogenous metastasis, the hematogenous dissemination of carcinoma is the most dangerous aspect of the disease, because it eliminates the surgeon's ability to control cancer through resection. The understanding of the mechanisms by which metastasis is established at the site of embolic lodgment is certainly necessary for comprehension of the pathogenesis.

The chief limitation to date for exploring the mechanisms of metastasis has been the lack of appropriate experimental models. One approach was that of Wood (23), who used microcinematography on rabbit ear chambers to elucidate the events in the establishment of V2 metastases. The delineated sequence of events was as follows: (a) tumor cell adherence to capillary endothelium, (b) thrombus formation with tumor cell entrapment and resultant endothelial damage, and (c) penetration of the damaged endothelium 1st by leukocytes and then by tumor cells. Although his sequence of events is not identical to the one described in this paper, Wood placed a time interval of 3 days for definitive establishment of micrometastases, a time period similar to the one described in this paper.

More detailed knowledge of these events should certainly be obtainable with the electron microscope, provided one has a suitable model for overcoming sampling difficulties. Ludatscher et al. (16) looked at Morris hepatoma emboli in pulmonary vessels of the rat with the electron microscope and concluded that they extravasated by an amoeboid process much like that of leukocytes.

Our experiments may be discussed in accordance with Roberts' (18) subdivision of the metastatic sequence into 4 parts.

**Embolization.** In this experiment, tumor cells were artificially introduced into the vasculature by parenteral injection, whereas this event generally occurs spontaneously in the disease process. Since the majority of tumor cells in a spontaneous primary cancer remain extravascular, this suggests to many investigators that in most cases the metastatic cell is a specialized one with respect to the majority of cells in the original tumor mass. This specialization is probably more than just a fortuitous selection based on juxtaposition to the vascular channels, since it is clear that many, if not most, tumor emboli do not become metastases. Thus it is not surprising to see a number of changes in Yoshida hepatoma cells while still emboli prior to invasion. This seems to represent a functional differentiation, but at least in this model it corresponds to a morphological dedifferentiation, i.e., the loss of an organized epithelial architecture with tight junctions and the development of amoeboid processes. Considerable intracellular morphological change occurs even earlier. These changes consist of enlargement of mitochondria, aggregation of ribosomes, accumulation of lipid, and synthesis of glycogen prior to any evidence of cell division, as extensively described previously (15). The peculiar architecture of the ascites islands seems to be one suited to ascites growth and not to metastatic invasion, and is accordingly reversible.

**Transportation.** Cells introduced into the chorioallantoic vasculature are collected into the umbilical vein and then go directly to the liver. The route by which naturally occurring emboli might get to the liver sinusoids may be more devious, depending on the primary site. At any rate the liver is one of the most common sites of extensive metastatic disease.

**Lodgment.** The process of trapping of tumor cells is difficult to evaluate from static 2-dimensional electron micrographs. The emboli are found abundantly in the liver before there is any evidence of tumor-endothelial interaction. Perhaps this is due to simple mechanical factors, considering the relatively large size of tumor emboli as compared with capillary diameters. In this model, however, many tumor emboli pass through the liver and metastasize to virtually every other organ in the embryo (10).

**Establishment.** In the chick embryo liver, the tumor cells do not pass through preexisting channels, but instead there is active destruction of the endothelial integrity. Through pseudopodal interaction, tumor cells can be seen peeling the endothelium away from the liver parenchyma, and indeed these endothelial fragments appear later to form the stroma of
the tumor. Other sites, however, show tumor cells surrounding intact sinusoids, suggesting that once out of a vascular space through destructive mechanisms, the tumor cells utilize adjacent intact vessels as their own capillaries. This is an obvious advantage to the tumor cells because they can maintain their blood supply. This may also represent an in vitro example of the “neoplastic blockade” demonstrated previously in vitro (13). By placing themselves between the liver cells and their vessels, the tumor cells would appear to get the 1st choice of nutrients and O₂ within the vessel. There must, however, be adequate perfusion of the underlying liver cells, because no necrosis of hepatocytes is apparent at 72 hr or even several days later, although liver cells may appear to be totally surrounded by tumor cells. Although there is at no time any active destruction of liver cells, either during or as a prerequisite to invasion, it is possible that tumor cells pinocytize material from intact liver cells. Such an ability, to tap the liver cells without destroying them, would suggest an elegant adaptation of the tumor cells to the host-parasite relationship which would permit a considerable lengthening of the duration of the relationship.

Yoshida Ascites Hepatoma 7974 is probably as different from human cancers as the chick embryo is from man. The sequence of events elaborated here is 1 plausible mechanism for the establishment of metastasis which is at least compatible with the human disease process and perhaps even provides some insight into the biological processes of host-tumor interaction which make cancer cells such efficient parasites.

REFERENCES

Figs. 1 to 2c. A series of micrographs of Stieve's fixed paraffin-embedded tissue showing the progression of the metastatic lesion from ascites tumor to well-established 72 hr metastasis.

Fig. 1. Donor ascites tumor (7 days old) in a Gelfoam sponge. AH 7974 grows predominantly as balls of cells in the rat peritoneum. Hematoxylin, X 250.

Fig. 2a. Tumor in 12-day-old chick embryo liver, 24 hr after i.v. inoculation. Balls of tumor cells are found in the sinusoids (arrows). Mitotic figures are absent. Hematoxylin, X 250.

Fig. 2b. Tumor in 13-day-old chick embryo liver, 48 hr after i.v. inoculation. Arrows, margins of individual metastases, which have at least doubled in size during the previous 24 hr. Numerous mitoses are present. Hematoxylin, X 250.

Fig. 2c. Tumor in 14-day-old chick liver, 72 hr after inoculation. Arrows, margins of 1 or more metastases. Mitotic figures are still numerous, and tumor now represents as much as 30% of the total liver in cross-section. Hematoxylin, X 250.

Fig. 3. Tumor in 13-day-old chick liver, 48 hr after inoculation. Hepatocytes (H) are dark; tumor cells are much lighter. Some tumor cells (Y) have elongated to line the sinusoidal space and surround the zone of tumor proliferation. Toluidine blue, X 1000.

Fig. 4. Metastasis at 72 hr. When compared with Fig. 3, this shows the establishment of an alternate architecture. Here, the growth is not intrasinusoidal. The lighter tumor cells are actively dissociating the liver parenchyma (H). Arrow, bile canalculus partly surrounded by tumor cells. Toluidine blue, X 1000.

Figs. 5 and 6. Donor ascites tumor (7 days old).

Fig. 5. Part of a ball of tumor cells from the rat peritoneum (see Fig. 1). Tight junctions are always present at the tumor-fluid interface (arrows). X 5500.

Fig. 6. Higher magnification of a junctional complex. Note the tight junction (arrow) at the surface of the tumor mass. There is also a desmosome here, but usually desmosomes are randomly distributed. X 26,500.

Fig. 7. Tumor embolus in a sinusoid (Ssin) 24 hr after inoculation. Tight junctions are still apparent at the tumor-fluid boundary (arrows). Note the absence of interaction with the endothelial cells (En), Kupffer cells (K), and a hepatocyte (H). There is contact here, but no evidence of interaction. X 8000.

Fig. 8. Higher magnification of a field similar to Fig. 7. The field shows the juxtaposition of a tumor cell (Y), a Kupffer cell (K), other sinusoidal lining cells (X), and a hepatocyte (H). There does not seem to be any interaction. X 17,000.

Fig. 9. At 48 hr, the tumor cell (Y) surface has become much more specialized. Compare with Fig. 8. Here, the tumor cell shows microvillus-like pseudopod formation, facing some hepatocytes (H). X 16,500.

Fig. 10. Tumor in the sinusoid after 48 hr. The tumor cells have dissociated from one another and most of the tight junctions are gone. One of the tumor cells has sent out a complex pseudopod (arrows) which is interdigitated with the fibroblast (F) and Kupffer cell (K) lining the hepatic parenchyma (H). X 7000.

Fig. 11. A high-power view of interactions similar to Fig. 10. Tumor cells (Y) have sent out tortuous pseudopods (arrows) which interact with sinusoidal fibroblasts (F) and which appear to contact the underlying hepatocytes (H). X 17,000.

Fig. 12. Tumor cells may also send out much larger pseudopods into the liver parenchyma. At this magnification, the pseudopod appears as a large vacuole within the liver cell. X 3000.

Fig. 13. Enlargement of the area marked off in Fig. 12. The pseudopod shows interlocking surface processes with the adjacent hepatocyte, while the tumor cell to the right does not. There are several rows of pinocytic vesicles within the pseudopod; fewer are seen in the tumor cell proper. X 10,500.

Fig. 14. Metastasis at 72 hr. This is the center of a metastatic focus. Note the lack of tight junctions between tumor cells. Arrows, fragments of sinusoidal lining cells which appear to have become the tumor stroma. X 9000.

Fig. 15. A normal sinusoid of 14-day-old chick embryo liver. The lining above the hepatocytes (H) consists of several cell types, including a fibroblast (F) and thin leaflets of endothelium (arrows). Note several discontinuities, and the thinness of the covering over the Disse space (Ds). The cell within the sinusoid is an erythrocyte (Er). X 9000.

Fig. 16. An invaded and surrounded sinusoid (72 hr). There is a tumor cell within and several tumor cells (Y) outside the intact lining of the sinusoid (arrows). There are many more fibroblasts (F) than normal, indicating a fibroblast hyperplasia. X 7500.

Fig. 17. Liver cells (H) and tumor cells (Y) in intimate contact with one another after 72 hr. There is no evidence of liver cell destruction. X 12,000.

Fig. 18. Liver cell (H) in contact with a tumor cell (Y). There is a slight thickening of opposing membrane faces, which may represent a kind of rudimentary junctional complex formation. X 22,500.

Fig. 19. Invagination of a liver cell (H) into a tumor cell (Y) channel. Arrow, pinocytic vesicle at the end of the channel; perhaps this represents incorporation of liver cell material by a tumor cell. X 22,500.
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