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Bone Resorption in Osteogenic Sarcoma—II. Resorption of the Bone Collagenous Matrix by Tumor Cells, Normal Fibroblasts and Macrophages*

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Abstract—*Three osteogenic sarcoma cell lines of human and canine origin were compared to normal fibroblastic cells and peptone-induced murine peritoneal macrophages in terms of bone collagenous matrix (BCM) resorption capacity. The dissolution of the BCM was measured in an* in vitro *system consisting of the tested cells and live or killed [^{3}H]-proline-labeled fetal mouse long bones. Experiments with osteogenic sarcoma cells revealed a paradoxical phenomenon indicating an inverse relationship between the number of tumor cells and the rate of collagen reorption from live bones. On the other hand, collagen matrix of devitalized bones, particularly those denatured by exposure to heat, is strongly resorbed by osteogenic sarcoma cells even in the presence of serum. Contrary to osteogenic sarcoma cells, normal fibroblasts do not resorb collagen from either live or killed bones, regardless of the devitalization method and culture conditions utilized. Macrophages resorb collagenous matrix from live bones, but their collagen resorption activity from devitalized bones depends greatly on choice of the incubation condition. Results have shown that osteogenic sarcoma tumor cells, when acting alone, may not have the capacity to destroy healthy bone. We suggest,therefore, that bone destruction seen in osteogenic sarcoma patients depends on the metabolic condition of the affected bone, and the interaction between the tumor and normal host cells and tissues.*

INTRODUCTION

THE DEVELOPMENT of osteogenic sarcoma is accompanied by variable damage to the adjacent normal bone [1]. In particular, the highly osteolytic forms of osteogenic sarcoma such as the 'telangiectatic type' present with scanty tumor osteoid but produce massive destruction of both medullary and cortical bone [2]. According to some investigators [3] such osteogenic sarcomas have a particularly grave prognosis, although this contention is not confirmed by others [4]. From the cellular and pathological points of view, the phenomenon of osteolysis may be related to an apparent strong invasiveness of the osteogenic sarcoma tumors.

Basic mechanisms of bone collagenous matrix (BCM) resorption are still not completely understood. The role of osteoclasts in this process has been questioned, because collagenous fibrils have not been seen within these cells and because collagenase activity of these cells is apparently low [5–7]. Macrophages were implicated in the resorption of BCM following observations that these cells phagocytoze collagen [8, 9] and also secrete collagenase into the extracellular media [10]. It has subsequently been shown that macrophage-like cells are an integral part of bone cellular population [11, 12] and participate actively in disruption of the bone-mineralized matrix and ingestion of the released debris [13].

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Involvement of osteogenic sarcoma cells in the resorption of the BCM has not been extensively studied. Nevertheless, intracellular collagen fibrils have been observed within the tumor cells in two out of six osteogenic sarcomas, as well as in other mesenchymal tumors examined [14]. It was not determined whether these collagen fibrils are of extracellular origin. Fibroblasts have been shown to both synthesize [15] and enzymatically degrade [16] collagen, especially in inflammatory connective tissue [17]. Thus it is accepted that fibroblasts possess the capacity to produce and degrade their own product. The phagocytosis of collagen fibrils was suggested as an alternative pathway for collagen degradation during wound healing [18], involution of post partum uterus [19] and periodontal disease [20, 21]. Nevertheless, the majority of findings which describe collagen phagocytosis concern periodontal ligament fibroblasts [22–25]. According to McArthur *et al.* [26] fibroblasts were found to be unable to resorb collagen from mouse calvaria.

This paper will describe collagen matrix removal from live and dead bones by three types of cells which exist within or in the vicinity of the osteogenic sarcoma tumor mass. Similar to the process of calcium resorption [27], degradation of the BCM is also dependent on the type and number of cells used, culture conditions and the physical state of the bone explant.

MATERIALS AND METHODS

Cell lines and culture media

Cell lines of osteogenic sarcoma derived from human (MG-63, G-292) and canine (D-17) tumors were maintained in F15 (MG-63, D-17) and McCoy's 5A media (G-292) (Gibco, Grand Island, NY) as described previously [27]. Two normal fibroblastic cell lines, one of murine origin (3T3-A31) and the other derived from human normal skin (F-18) were grown in H21 and F11 media (Gibco) respectively. All media were enriched with 10% heat-inactivated, low-endotoxin, fetal calf serum (HI-FCS) (Sterile Systems, Inc., Logan, UT) and an antibiotic mixture (100 U penicillin and 100 μg streptomycin/ml; P/S).

Peritoneal macrophages

Peritoneal macrophages were selected by the adherence method from peritoneal exudate cells (PEC) induced in Swiss Albino mice by an i.p. injection of peptone nutrient broth (1 ml/mouse; BBL Co., Cockeysville, MD) three days previously. PEC were collected into Dulbecco's phosphate-buffered saline (DPBS), plated (M-alpha medium without nucleosides, 10% HI-FCS, P/S; GIBCO) into plastic culture dishes (Nunc; Vangard International, Neptune, NK) and cultured in an incubator (37°C; 5% CO_2–95% air; 95% humidity). The medium was changed after 2 hr, and free non-adherent cells were discarded. Fresh medium was added and macrophages were cultured for an additional several hours, or overnight, before collection with a rubber policeman. Cells were counted in a hemacytometer and were seeded in the desired quantity into a 24-well microplates (Linbro, New Haven, CT).

Bone organ explants

Pregnant Swiss albino mice (Hilltop Laboratories, Inc., Washington, PA) were injected via the tail vein on day 18 or 19 of gestation with 200–400 μCi of [^{3}H]-proline (5 Ci/mmol, Brea, CA). Tibiae (T) and humeri (H) with labeled collagenous matrix were dissected from fetal or newborn (less than 1 day old) mice. The cartilagenous ends of these long bones were removed and the calcified diaphyses were carefully isolated under a dissecting microscope. The bones were then preincubated overnight in BGJ_b media containing 10% HI-FCS and P/S to remove most of the unincorporated label.

The assay of the collagenous matrix resorption

Bone resorption assays were performed according to modifications of established methods [28, 29], as reported elsewhere [27]. Live or devitalized bone explants were incubated for 4 days in Linbro culture plates with (experimental cultures) or without (control cultures) the specified number of tested cells in BGJ_b or other medium (see Results) containing 10% HI-FCS and P/S. For each experiment only bones from one litter were used and explants were randomly distributed between control and experimental cultures. At the end of the culture period the media were collected, cleared by centrifugation and the released radioactivity determined. Results are expressed as the ratio of radioactivity released in experimental cultures to the mean of radioactivity released by control bone cultures. At least three separate experiments, with triplicate assays, were performed and the results were evaluated by Student's *t* test.

Control experiments have shown a linear correlation between released radioactivity and the amount of the free hydroxyproline present in the medium. This is in accordance with similar tests performed by others [30, 31]. For this reason a less cumbersome method of determining collagen resorption, by monitoring released radioactivity, was used throughout this investigation. Total radioactivity incorporated by the bones was determined by complete hydrolysis of explants in 6 N HCl for 18 hr at 118°C. This was done to make certain that the amount of released radioactivity

did not exceed approximately 75% of the total radioactivity incorporated. This precaution is of importance when data are expressed on the basis of released radioactivity instead of quantitative determinations of free hydroxyproline.

Bone devitalization

Previous experiments [27] indicated that bones devitalized by various means do not respond equally to effector cells in terms of calcium resorption. Isolated bones were devitalized in several ways to obtain explants with various degrees of collagenous matrix denaturation as follows: freeze–thaw cycles (three times in saline) with the temperature kept below 4°C during the entire procedure, u.v. irradiation (a total of 8 hr with bone explants placed on DPBS-soaked filter papers), 45°C incubation (in DPBS for 30 min) and boiling in water (10 sec). Histological observations and cell-outgrowth experiments [32] confirmed the lack of viable cells in devitalized explants. In addition, none of the devitalized bones responded to the parathyroid hormone or prostaglandin E_2.

RESULTS

Cell-number-dependent resorption of BCM

The release of radioactivity from [^{3}H]-proline-labeled living bones was stimulated moderately by osteogenic sarcoma cells, provided they were seeded at low numbers in the mixed bone organ–tumor cell cultures (Fig. 1a). However, it was observed that the process of matrix resorption was either not affected or was suppressed in the presence of higher numbers of tumor cells. In contrast, the collagen resorption from 100°C-killed bones was significantly promoted by osteogenic sarcoma cells (Fig. 1b). Under these conditions release of radioactivity was strongly dependent on cell number and resulted in dramatic collagenous matrix dissolution by confluent tumor cells (10^5 cells seeded at day 0). Under described experimental conditions, normal fibroblasts failed to promote or inhibit BCM dissolution from live bones (Fig. 2a). Release of ^{3}H-labeled material from 100°C-devitalized bones was also not affected by either of the fibroblastic lines tested (Fig. 2b).

Macrophages caused the release of [^{3}H]-proline labeled materials from living bone (Fig. 3a), a phenomenon dependent on the number of cells initially seeded in the cultures. These cells were without effect in assays containing bones devitalized by exposure to 100°C heat (Fig. 3b).

Effect of media pH

Co-culture of isolated bones with various cell types results in progressive acidifying of the media pH. In the first paper of this series [27] it was established that various metabolic products from our mixed cultures of bone explants and tumor cells acidify the culture medium and this, in turn, contributes to the calcium resorption. In contrast to the experiments with ^{45}Ca-labeled bones, daily additions of citric acid (10 μl of

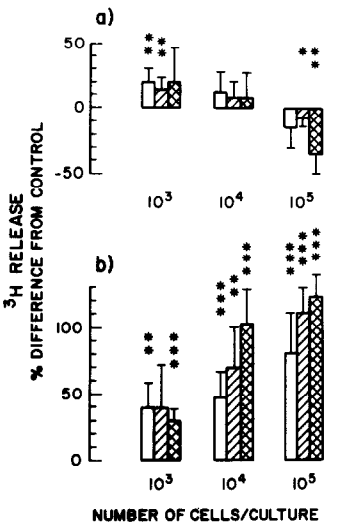

Fig. 1. Cell-number-dependent release of radioactivity from [^{3}H]-proline-labeled bones by osteogenic sarcoma cells. (a) Live bones. Osteogenic sarcoma, MG-63 □, G-292 ▨ and D-17 ■ cells were co-cultured with [^{3}H]-proline-labeled fetal murine bones (tibiae, humeri) in 1 ml of BGJ_b medium containing 10% HI-FCS, as described in Materials and Methods. Results were expressed as a percentage of the control (± S.E.). Statistical difference from the control: P >0.05 (*), P >0.01 (**), P >0.001 (***). (b) 100°C heat-killed bones. All other procedures as in (a).

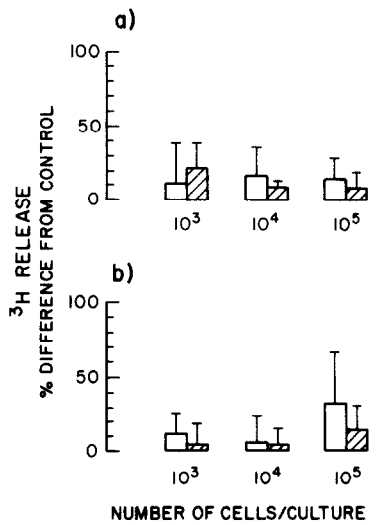

Fig. 2. Cell-number-dependent release of radioactivity from (a) living and (b) 100°C-devitalized [^{3}H]-proline-labeled bones by fibroblastic cell lines. Human skin fibroblasts F-23 □ and established cell line of normal mouse fibroblasts 3T3-A31 ▨ were used in these experiments. The assays were performed as described under Fig. 1 and Materials and Methods. Differences between the treatments and the control values were not statistically significant.

0.15 N citric acid/ml) or bicarbonate (0.4 N sodium bicarbonate; 10 μl/ml) caused no detectable change in the amount of released radioactivity from [^{3}H]-proline-labelled explants (Table 1). Table 1 also demonstrates that the pH of mixed cultures could be maintained near control values with additions of bicarbonate. Such adjustments of pH had no effect on the amount of the tumor cell-mediated release of radioactivity.

Effect of media and method of devitalization on the resorption of BCM

The control experiments suggested that the rate of collagenous matrix resorption would depend on the culture conditions, type of effector cells and physiochemical state of the bone collagenous matrix. In *in vitro* conditions the metabolic state of cells and organs can be modified by culture media and various supplements. The status of the collagenous matrix can be altered by physical treatments of explants which either preserve or denature collagen fibrils.

Experiments have shown that an initial seeding of 5×10^4 cells will result in a confluent layer on day 3. At this density, tumor cells had no effect on collagen resorption from live bones, regardless of the media utilized (AP, F15 or BGJ_b) (Fig. 4). However, when devitalized bones were employed, the type of medium made a significant impact on the rate of BCM resorption. Co-culture of

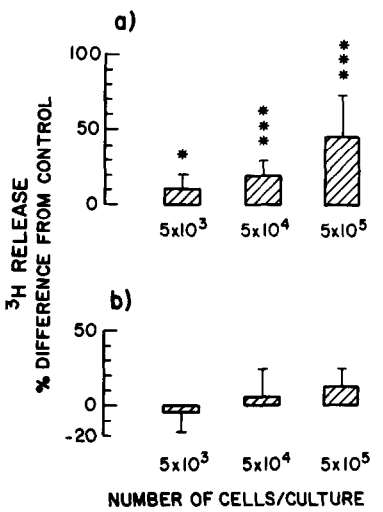

Fig. 3. Cell-number-dependent release of radioactivity from (a) living and (b) 100°C-devitalized [^{3}H]-proline-prelabeled bones by mouse peritoneal macrophages. Mouse peritoneal macrophages were elicited by an intraperitoneal injection of peptone nutrient broth. Macrophages were seeded in mixed bone organ-cell cultures, as described in Materials and Methods and under Fig. 1. Statistical significance is as noted in Fig. 1.

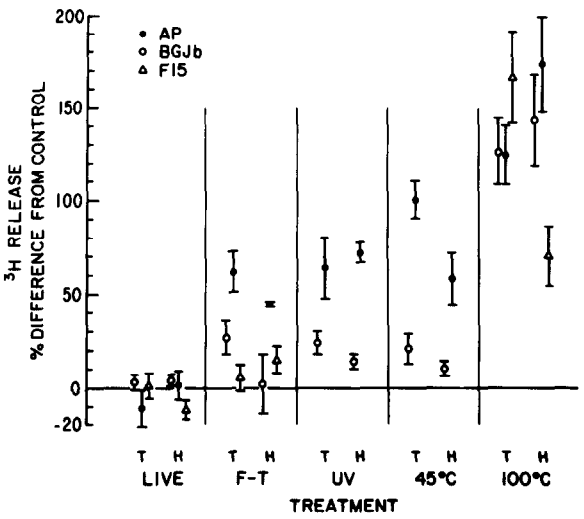

Fig. 4. Effect of media and methods of devitalization on resorption of the BCM by MG-63 osteogenic sarcoma cells. The MG-63 cells were seeded at 5×10^4 cells/well at day 0 in three different media containing 10% serum (HI-FCS) and antibiotics (P/S): AP (•), F15 (Δ) and BGJ_b (○). Live or devitalized tibiae (T) and humeri (H) (see Materials and Methods) were placed in cell cultures on day 0; all other procedures were as in Materials and Methods and Fig. 1. Amount of released radioactivity was determined on day 4, with results expressed as mean percentage difference (± S.E.) from control cultures containing bone explants only.

Table 1. pH effect on BCM resorption by D-17 osteogenic sarcoma cells

Treatment	dpm released	T/C	pH
Control—live	587 ± 47	1.00 ± 0.08	7.35 ± 0.05
+D-17 cells	613 ± 76	1.05 ± 0.13	7.25 ± 0.03
+D-17 cells + $NaHCO_3$	560 ± 58	0.96 ± 0.10	7.39 ± 0.05
Control—killed (100°C)	302 ± 15	1.00 ± 0.05	7.47 ± 0.03
+ citric acid	313 + 34	0.89 ± 0.11	6.90 ± 0.09
+ $NaHCO_3$	292 ± 56	0.98 ± 0.18	7.50 ± 0.06
+D-17 cells	717 ± 94	2.38 ± 0.31	7.30 ± 0.07
+D-17 cells + $NaHCO_3$	630 ± 86	2.08 ± 0.28	7.45 ± 0.05

D-17 tumor cells (5×10^4 cells/well) were seeded in 1 ml of media (AutoPow, 10% HI-FCS, P/S) to bone cultures on day 0. The final pH (day 4) in such mixed cultures was adjusted to near the control (wells with bones only) value by daily additions of 10 μl of 0.4 N $NaHCO_3$ on days 1, 2 and 3. Of the total 1 ml samples, 0.2 ml were used for determination of radioactivity. Data are presented as weighted controls and as the ratio of treatment to control (T/C) values (± S.D.).

devitalized bone explants with tumor cells in AP medium resulted in significant radioactivity release. The agent responsible for this effect does not appear to be secreted in assays performed in either F15 or BGJ_b media. The rate of resorption was always highest in experiments with 100°C-treated bones, regardless of the media utilized.

Several lines of normal fibroblasts were tested under the same conditions. Both F-18 and 3T3-A31 cells were found unable to resorb BCM from live, as well as devitalized, bones (Fig. 5a).

Macrophages elicited by peptone injection (Fig. 6) induced resorption of collagen from live

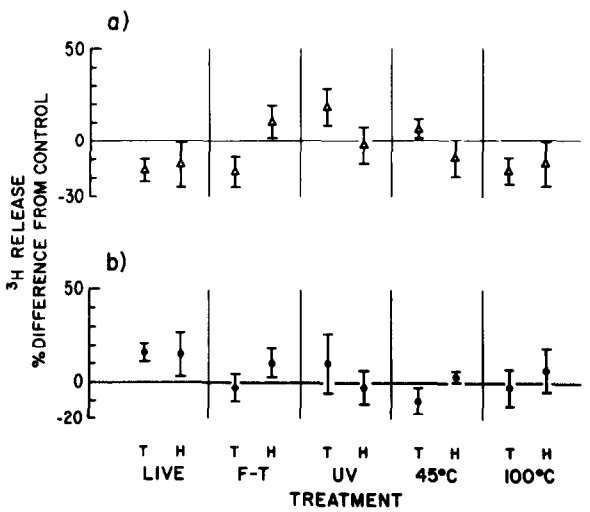

Fig. 5. Effect of methods of devitalization on radioactivity release from [^{3}H]-proline-prelabeled bones by normal fibroblasts. The live or devitalized bones (T, tibiae; H, humeri) were cultured together with either F-18 normal human skin fibroblasts or 3T3-A31 normal murine fibroblastic cells. The initial density of the cultured cells was 5 × 10^4/well in F11 and AP media, both containing 10% HI-FCS, P/S. All other procedures were the same as in Fig. 4.

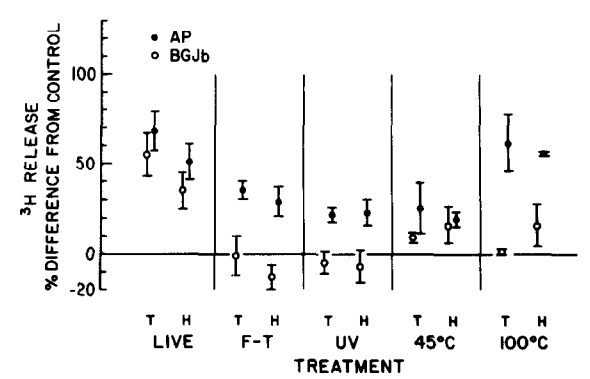

Fig. 6. The effect of media and methods of devitalization on resorption of the BCM by peptone-elicited macrophages. Macrophages were isolated as described in Materials and Methods and under Fig. 3. Cells were then seeded on day 0 at 5 × 10^5 cells/well either in BGJ_b (○) or AP (•) media, each containing 10% HI-FCS, P/S. The macrophages were co-cultured either with tibiae (T) or humeri (H); control cultures contained no cells. All other procedures as under Fig. 4.

bones in both media tested (BGJ_b and AP). However, only AP medium supports macrophage-mediated BCM resorption from devitalized bones. As with tumor cells, a pronounced effect was seen in cultures containing 100°C-devitalized bones.

DISCUSSION

Spatial and temporal relationships between the resorption of bone mineral and collagenous matrix has yet to be fully explained. Heersche [33] advanced an hypothesis suggesting a two-stage bone resorption process which functions in normal physiological conditions. According to this theory the calcium and collagen are resorbed by two different sets of cells: osteoclasts and macrophages or fibroblasts respectively. The process of bone collagen removal under pathological conditions, such as primary or secondary bone tumors, is even less well understood. The osteogenic sarcoma cells, fibroblasts and macrophages are abundant cellular components present within, or in the vicinity of, the osteogenic sarcoma tumor mass. In this study we examined the collagen degradative properties of these cells using both live and devitalized bones. This is the first step toward understanding possible interactions between tumor cells and various normal cells in tumor-associated bone damage.

None of the tested osteogenic sarcoma cell lines efficiently resorbed BCM from live bones. In fact, an inverse relationship was found between the amount of collagen resorption and the number of tumor cells seeded into the culture wells. With a seeding of 10^5 cells per culture, this trend resulted in an inhibition of released radioactivity below control level. Since similar results could be obtained with conditioned media from osteosarcoma cell cultures [Novak, in preparation], the data cannot be explained by competition for nutrients between the cancer cells and bone explants. Therefore the results suggest the production of bone resorption inhibitor by the tumor cells in culture. Normal fibroblasts at any initial cell density neither stimulated nor inhibited collagen bone resorption from live or devitalized bones. This lack of activity does not correspond with reports describing active collagen phagocytosis by cultured periodontal ligament fibroblasts [23, 24] and human gingival fibroblasts [25] *in vitro*. Additionally, Svoboda and Deporter [34] found fibroblasts to be more effective than macrophages in phagocytozing collagen fibrils. Nevertheless, we found peptone-elicited macrophages to be the only tested cells capable of significant BCM resorption. It was demonstrated that bone tissues are chemotactic for both monocytes [35] and tumor cells [36]. Therefore this property may play a role in

collagen removal by extracellular collagenolysis [10] and/or phagocytosis [8]. It is also possible that macrophage-mediated collagen resorption from live bones could be caused by secretion of prostaglandins [37, 38]. Prostaglandins are known to induce osteoclastic resorption of both calcium and BCM [39].

Specific bone collagenase [40, 41] as well as collagenases of extraskeletal origin [42] and lysosomal enzymes [43, 44] have been implicated in collagen matrix resorption. Collagenases are found in the biological fluids, usually in an inactive form as proenzyme [16] or as complexes of enzyme and inhibitors. Various inhibitors of collagenases have been identified from bone [45], cartilage [46], serum [47] and other tissues [48]. Despite the inclusion of serum in all our culture media, we demonstrated significant resorption of collagen under several experimental conditions.

Macrophages and tumor cells participate in yet another way in collagen resorption from cultured bones. This other mechanism directly affects devitalized bones, with either native or denatured collagenous matrix. It is most pronounced when cultures are maintained in AP media. The strong ability of osteogenic sarcoma cells to resorb 100°C-denatured collagenous matrix [49] was directly related to cell number. The same results were obtained in experiments with conditioned media from tumor cells (unpublished results). According to Milsom *et al.* [44], preparation of crude lysosomal enzymes could partially depolymerize native collagen fibrils. Such a depolymerization process may proceed slowly at a neutral pH but becomes optimal in acidic conditions, when it is accompanied by release of short, dialyzable peptides. Nevertheless, we found that under our experimental conditions the rate of collagen resorption is not affected by pH changes within the range of 6.9–7.5. This result is similar to the conclusions by Dominguez and Raisz [50] and strengthens the contention that tumor cells [31] as well as some normal cells [51] may affect bone resorption processes by at least two different mechanisms. First, by modifying the activity of osteoclasts, and second, by a direct mechanism, independent of both osteoclasts and pH changes.

Alternatively, gelatinase, a product of leukocytes [52] and fibroblasts [53, 54], has been shown to degrade heat-denatured collagen. Our preliminary results with conditioned media and specific inhibitors (unpublished results) indicate that this may be the activity responsible for osteogenic sarcoma degradation of heat-denatured bone collagenous matrix.

In conclusion, we have found that each of the cell types associated with osteogenic sarcoma manifests a characteristic pattern of bone calcium release [27] and collagenous matrix resorption. Our results indicate that the bone destruction seen in the pathological specimens does not appear to be mediated by osteogenic sarcoma cells alone. It is therefore conceivable that the tumor-associated bone damage is a result of cellular interactions between tumor and normal cells.

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