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Intercellular Junctional Specializations in Human Basal Cell Carcinoma

A Freeze-Fracture Study*

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Summary. Intercellular junctions of various types were found on the membrane fracture faces of human nodular basal cell carcinoma (BCC) cells. The junctional types represented include desmosomes, tight junctions, and gap junctions. A semiquantitative comparison of undifferentiated and differentiated nodular BCC showed that gap and tight junctions were observed on all exposed membrane fracture interfaces of the differentiated tumors, while only fifty six per cent of the membrane interfaces of the undifferentiated tumor exhibited similar junctional specializations. These membrane specializations may be a partial reflection of differentiation among the different types of BCC and their contribution to the less invasive character of nodular BCC cannot be ruled out.

Key words: Freeze fracture – Desmosomes – Gap junctions – Tight junctions.

Introduction

There has been an increased interest in the junctional associations of tumor cells in recent years. Certain intercellular junctions (desmosomes, tight junctions) contribute to cell-to-cell adhesiveness while others (gap junctions) may also be involved in growth regulation (Loewenstein, 1968; Loewenstein, 1968; Gilula, 1977; Pitts, 1977; Revel, 1978). Therefore, alterations in junctions could conceivably account for or contribute to the behavior of malignant tumors. The abundance of studies and case reports in the literature which include material on intercellular junctions may lead one to believe that their occurence in tumors displaying different growth patterns has been well documented. However, almost

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all of these studies utilized the thin section technique and contained mostly descriptive information. There are only a few reports in the literature which have used the freeze-fracture technique to study intercellular junctions in tumors (i.e. Alroy and Weinstein, 1976; Sinha et al., 1977; Banner et al., 1978).

The basal cell carcinoma (BCC), a common skin tumor in humans, is characterized by slow growth and local invasion. Metastasis is exceptionally rare (Safari and Good, 1977). It also shows a tendency for differentiation toward different adnexal structures resulting in different histologic appearances of the tumor (Wade and Ackerman, 1978). All these characteristics make BCC a good subject in which to investigate the occurrence, structure, and possible alterations of intercellular junctions in different phases of tumor invasion.

Investigation of intercellular junctions of normal human skin, utilizing thin sections (Breathnach, 1971) and freeze fracture replicas (Caputo and Peluchetti, 1977; Elias, and Friend, 1975; Hashimoto, 1973), has already been done. Relatively few investigations, however, were performed on the intercellular junctions of BCC, and the pertinent thin section electronmicroscopic literature is well summarized by Lapis (1976). Flaxman (1972) and Flaxman and Cavoto (1972), using thin section and electrophysiological methods, have shown that BCC contain tight and gap junctions, however, the majority of their observations were made on tumor explants. Since it is well known that BCC undergo morphological and behavioural alterations in vitro (Flaxman and Van Scott, 1968; Cooper and Pinkus, 1977) the results of these two studies and those of our present investigation are not comparable. To our knowledge there are no reports describing the intercellular junctions of BCC using the freeze fracture technique.

In this paper we will document the presence of gap and tight junctions in BCC with nodular growth patterns and attempt to correlate their number with tumor differentiation.

Materials and Methods

Tumor tissues removed from patients, using Moh's fresh tissue control technique (Mohs, 1976), were immediately fixed in 2.5% glutaraldehyde in 0.1M cacodylate, 0.1M sucrose. Light microscopic sections prepared from these tissues served as a control for selection of appropriate portions of the tumor for thin section electron microscopy and freeze fracture studies.

The methodology used for the thin section and freeze fracture studies was the same as previously reported (Meyer et al., 1977). Out of the 34 tumors received, 13 were suitable for thin section study and only four for the freeze fracture study. The applicability of the freeze fracture method for studying the intercellular junctions of BCC was limited by the size of the tumor received. Tumor nodules incorporated in the study had to be at least $1 \times 1 \times 1$ mm in size.

For the purpose of quantitating gap and tight junctions interfaces were selected that were at least 58 μm^2 (area of field at $\times 11{,}000$) in order to avoid the counting of cell processes and other small interfaces. The fractured membrane face was counted only if there was an indication of another cell present, that is a change in the fracture plane from the E face to the P face or visa versa (a "step"). The interfaces were then classified on the basis of the presence and number of junctional foci. A *junctional focus* may contain from one to many individual junctions and also different types of junctions that are confined to a recognizably separate area of the interface. The type of junctions in the foci included 1) tight junctions only 2) tight junctions in close association with small gap junctions 3) as in (1) and (2) but with the addition of solitary gap junctions 4) solitary gap junctions only (see Fig. 1c).

Because membranes are split during the freeze fracture process two membrane fracture faces from the hydrophobic interior of the membrane are seen. In the case of the plasma membrane, the half closest to the cytoplasm is labeled as the P or protoplasmic half and the half closest to the extracellular space is labeled as the E or exoplasmic half (Branton et al., 1975). In all electronmicrographs used in this report the direction of platinum shadowing is approximately from the bottom unless indicated by an encircled arrow.

Results

Light Microscopy

The tumors were classified light microscopically on the basis of the presence or absence of infiltration and the type of differentiation (Sloane, 1977; Wade and Ackerman, 1978). Most tumors showed nodular growth with some form of imperfect differentiation towards adnexal structures. "Undifferentiated" nodular forms and "pure" infiltrative forms were rarely seen.

The tumors used for the freeze fracture study were BCC with nodular growth patterns. One large tumor (approximately $13 \times 13 \times 3$ mm) showed confluent nodular subepidermal growth and was extensively sampled using the freeze fracture technique. The outer cell layers had the typical pallisading appearance while the inner cell mass consisted mostly of round cells. Areas with a focal spindle appearance and a few large atypical cells were also seen. No sign of necrosis or evidence of differentiation toward adnexal structures was observed (Fig. 1a). Three other tumors with nodular growth patterns and otherwise characteristic histologic appearance of BCC showed definite signs of differentiation toward adnexal structures (Fig. 1b). Areas of squamous "differentiation" and growth patterns resembling trichofolliculoma were also observed. Multiple nodules of these three tumors were examined using the freeze fracture technique.

Freeze Fracture Study

The plasma membrane fracture interfaces (see materials and methods) of BCC cells contained desmosomes, tight junctions, and gap junctions. The membrane interfaces were classified on the basis of the presence and number of junctional foci. A junctional focus may contain from one to many individual junctions that are confined to a recognizably separate area of the interface (Fig. 1c) The morphology of the junctions seen on freeze-fracture replicas was similar in all tumors examined in this study.

Desmosomes were the most obvious structures on the membrane fracture surfaces of basal cell carcinoma cells, and were observed on all of the membrane fracture faces included in this study. They were commonly recognized on both P and E membrane fracture faces as round or elongated forms containing irregularly shaped particles and filaments (Fig. 2a). An elevation of the P membrane fracture face (Fig. 2b) and a corresponding depression of the E membrane fracture face (Fig. 2c) at the site of the desmosomes was oftentimes noticeable. Hemidesmosomes were not observed in our freeze fracture replicas.

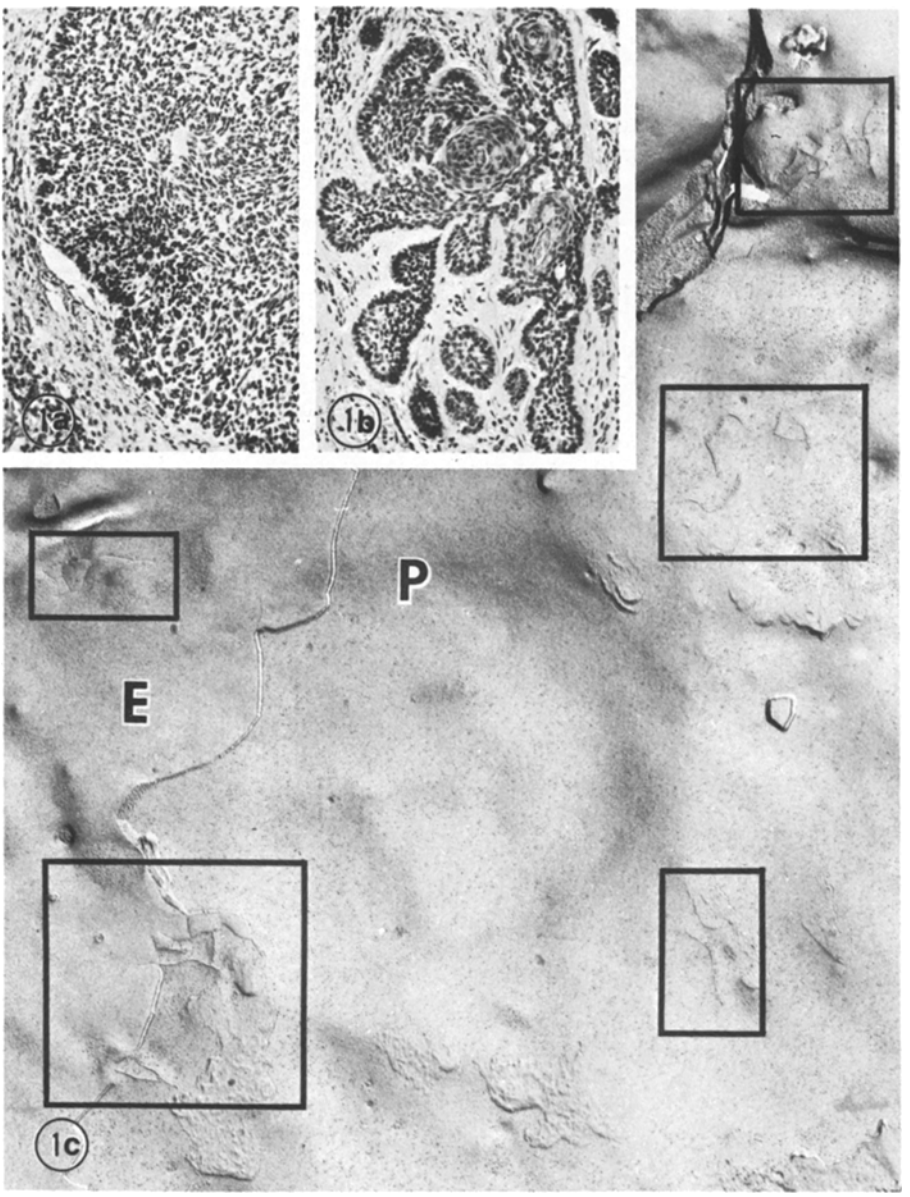

Fig. 1. a A part of a nodular basal cell carcinoma (BCC) which shows no signs of differentiation toward adnexal structures. ×200. **b** A portion of a nodular BCC which exhibits differentiation toward adnexal structures. ×200. **c** Low magnification membrane fracture interface from a differentiated tumor contains a heterogenous collection of junctional profiles. Junctional foci (*enclosed areas*) contain from one to many individual junctions that are confined to a recognizably separate area of the interface. P (*P*) and E (*E*) membrane fracture faces are labeled. ×20,400

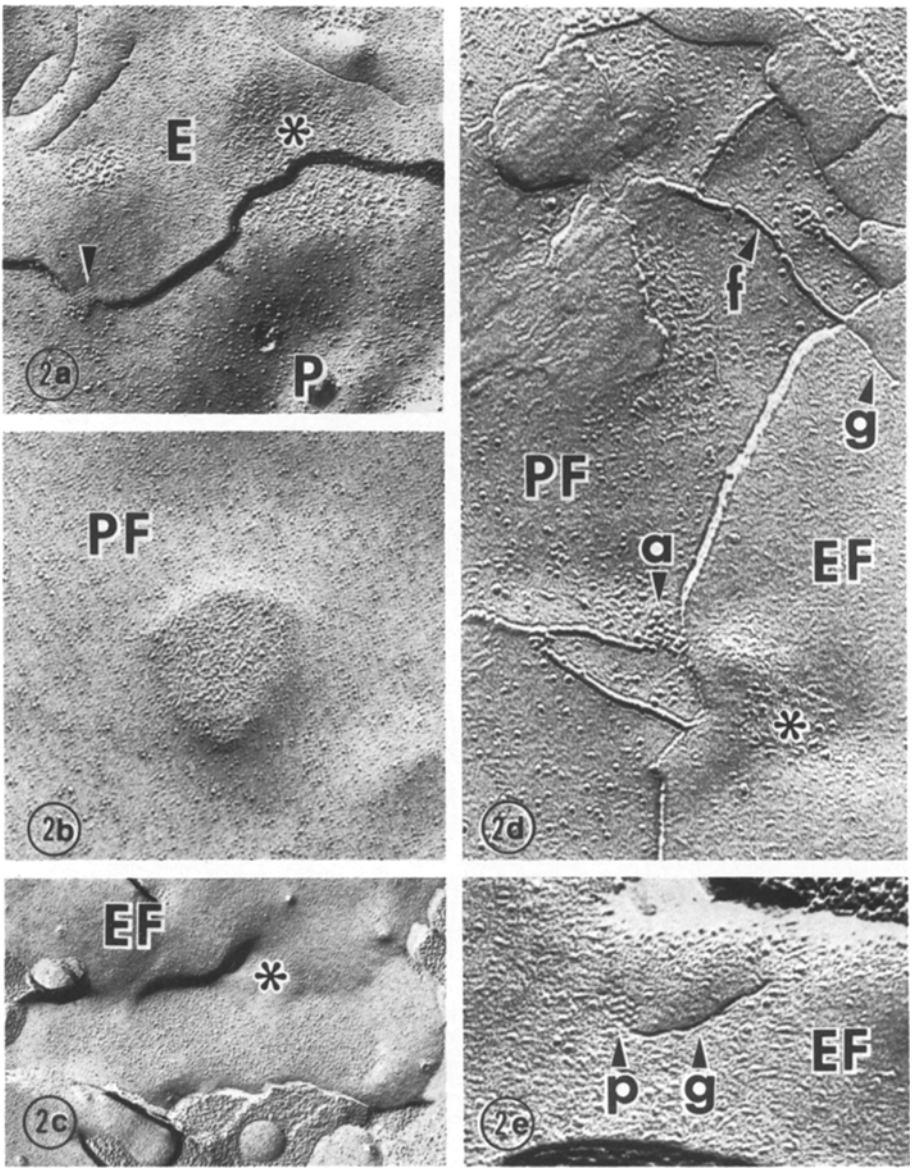

Fig. 2. a Desmosomes (*asterisk*) were recognized on both the E (*E*) and P (*P*) membrane fracture faces as round or elongated forms containing irregularly shaped particles and filaments. A normal intercellular space is oberserved at desmosomal sites. In contrast, the nearby gap junction (*arrowhead*) reveals uniform complementary particles and pits in transitional areas and a reduced intercellular space. ×36,000. **b** Desmosomes often occupied elevated regions on the P membrane fracture face (*PF*). ×51,000. **c** On the E membrane fracture face (*EF*) corresponding depressions with the characteristic desmosomal structure were frequently observed (*asterisk*). **d** The complementarity of tumor cell tight junctions can be shown in areas where the P face (*PF*) fibril (*f*) of one cell is seen to be continuous with an E face (*EF*) groove (*g*) in the adjacent cell. Particle aggregates (*a*) in close association with the tight junction fibrils were common. A desmosome (*asterisk*) is also labeled. ×84,000. **e** The finding of corresponding E face (*EF*) tight junction grooves (*g*) in close association with aggregates of pits (*p*) indicates that these small assemblies of particles or pits probably represent gap junctions. ×120,000

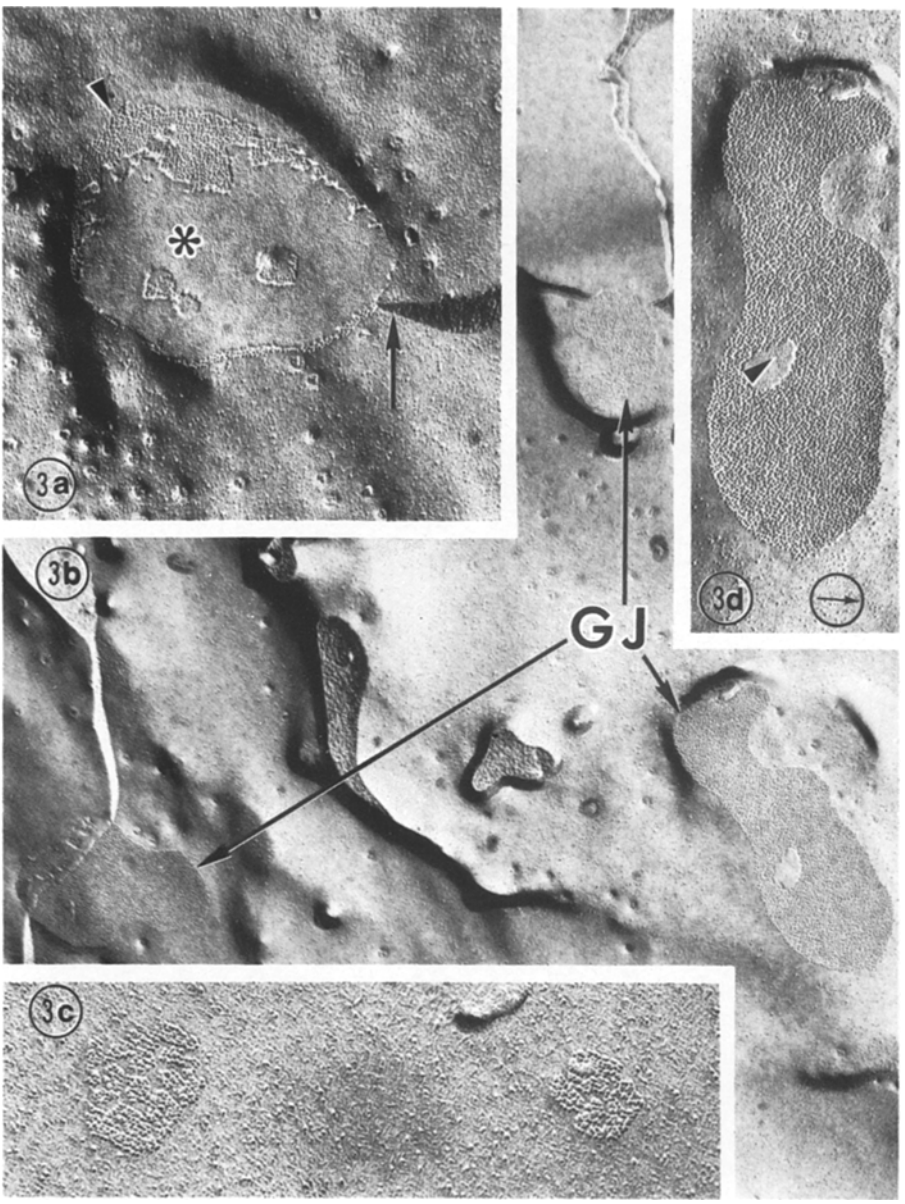

Fig. 3. a This micrograph illustrates the complementarity of gap junctions found between tumor cells. Fracture face transitions within this junction reveal both pits (*asterisk*) and particles (*arrowhead*). Note the characteristic narrowing of the intercellular space (*arrow*). ×45,000. **b** The most common type of gap junctions (*GJ*) seen in this study is illustrated by the tightly packed structures seen here. ×27,000. **c** There was some variation noted in the arrangement of particles within gap junctions. This micrograph illustrates a domain pattern, that is, small particle aggregates within the junction that are separated from one another by particle free areas. ×55,700. **d** This illustration is an enlargement of one of the gap junctions in Figure 3b and demonstrates that some of these junctions can attain considerable size. A small piece of E membrane fracture face generated within this junction contains the expected pits (*arrowhead*). ×40,500

Table 1. Basal cell carcinoma (freeze fracture)

	Interfaces counted[a] (Number)	Interfaces with junctional foci		Average no. of foci per interface
		No.	%	
Nodular undifferentiated	99	56	57	1.4
Nodular differentiated	38	38	100	4

[a] Interface is defined as 58 μm^2 of fractured membrane face that has an indication of another cell present

Tight junctions were usually characterized as short fibrils on the P membrane fracture face (Fig. 2d) or as distinct grooves in the E membrane fracture face (Fig. 2d and e). However, occasional, rather long, meandering tight junctional profiles were noted. Although individual tight junction strands were often clustered together (Fig. 1c and 2d) no structural orientation with respect to one another could be discerned.

Gap junctions were identified frequently on the P face as packed aggregates of particles (Fig. 3a–d) and on the E face by ordered arrays of pits (Fig. 2a, 2e, 3a, 3d). The complimentarity of these structures is revealed when fracture face transitions occur within the junctions (Figs. 2a, 3a, 3d). There was some variation in the arrangement of the particles; some were tightly-packed, while others exhibited a domain pattern (Fig. 3c). The junctions were found as solitary structures, and in very close association to tight junction maculae (Fig. 2d and e).

A semiquantitative study was performed on multiple tumor nodules from four specimens (Table 1). The results show that tumors with signs of differentiation contained junctional foci (Fig. 1c) on *all* of the observed membrane interfaces. In contrast, the undifferentiated tumor exhibited junctional foci on fifty six per cent of the observed membrane interfaces. Furthermore, the differentiated tumors had almost three times as many junctional foci per interface as the undifferentiated one. Two other observations, not evident in the table, include the fact that in the undifferentiated tumor the junctional foci usually contained only a single tight junction strand and that no solitary gap junctions were seen. In comparison, the junctional foci of the differentiated tumors most often exhibited many individual junctional elements and solitary gap junctions were common.

Thin Section Study

The thin section technique was used mainly to assess the viability of the tumor tissue used in the freeze fracture technique. Gap and tight junctions, although seen, were not common using this method, in contrast to their frequent occurrence in freeze fracture material.

Discussion

Because the presence of cell junctions can be of considerable value in the differential diagnosis of certain tumors, their description is included in an extensive number of studies and case reports. However in their recent review of the ultrastructural literature concerned with intercellular junctions in solid tumors Weinstein et al., (1976), noted that although "cell junctions are mentioned in many pathology papers, only a few contain quantitative information on specific types of cell junctions". It was concluded that the reason for this lack of data mainly resulted from the fact that cell junctions, especially gap junctions, are difficult to identify in thin sections even under the best of standard preparatory procedures. With regard to BCC, the small size and the association of different types of cell junctions present additional difficulties in documentation using the thin section technique. The freeze fracture technique provides positive identification of the specific type of cell junction, information on the association of different junctional types, and is not limited by the size of the junction. Thus, as the freeze fracture technique has been the method of choice in the study of intercellular junctions in normal tissue, it is also the definitive manner in which to establish their distribution and relative abundance in tumors.

Desmosomes seen in freeze fracture replicas were generally numerous in all samples studies, and revealed form and structure similar to that observed in the normal skin (Caputo and Peluchetti, 1977), and in the normal oral epithelium (Shimono and Clementi, 1976). Desmosomal modifications of the type described during early keratinization of normal skin (Caputo and Peluchetti, 1977) and in the oral epithelium (Shimono and Clementi, 1976) were not noted although one tumor showed signs of squamous differentiation. The desmosomes observed in all samples of basal cell carcinoma fulfill the criteria of spot desmosomes. The spot desmosome provides direct mechanical coupling between the cytoplasmic tensile skeletons (tonofilaments) of adjacent epithelial cells through transmembrane protein linkers (Staehelin, 1974). The freeze fracture structure of the desmosomes reveals irregularly fractured particles which are seen on both the E and P membrane fracture faces. Most of these particles are probably not integral membrane proteins, but rather the filamentous "transmembrane linkers" that have been broken off and deformed during fracturing process (Staehelin, 1974).

Gap junctions in normal skin have been observed previously only in the basal and intermediate cell layers (Caputo and Peluchetti, 1977; Hashimoto, 1973). They were, however, small in size, very rarely encountered, and no association between gap junctions and tight junctions was mentioned.

The occurrence of a considerable number of gap junctions between the BCC cells indicates the likelihood of direct intercellular communication among these tumor cells. It has already been shown that gap junctions provide an approximately 20 Å-in-diameter connecting channel between neighboring cells (McNutt and Weinstein, 1970). Through these channels a wide range of molecules, up to a certain size (1200–1900 daltons, Simpson et al., 1976), may pass directly from one cell to another. Of particular importance would be the exchange of "informational" molecules which might affect growth control and differentiation (Revel, 1978). A recent report (Lawrence et al., 1978) of an

exchange of cAMP is therefore especially interesting. Additionally it has been proposed that growth control and differentiation are themselves related, with differentiation being more likely to occur in a slower growing population of cells (Sheridan, 1976). In BCC gap junction mediated communication may allow the individual tumor nodules to regulate their own growth and differentiation toward different adnexal structures. The reason for the slow enlargement of BCC has not yet been satisfactorally explained although it has been suggested to result from a close balance between mitosis and cell death (Weinstein and Frost, 1970; Kerr and Searle, 1972). In a review of 160 cases we have found only occasional mitoses and then almost exclusively in undifferentiated tumors. In any event kinetic studies (Weinstein and Frost, 1970) indicate that the S phase in BCC is significantly longer than the normal epidermis from which it originates and therefore BCC is at least a *relatively* slow growing cell population. Although certain undifferentiated, rapidily growing, transformed cells have been shown to possess functional gap junctions (Johnson and Sheridan, 1971, Pinto da Silva and Gilula, 1972) it may be that those cells have some defect in their ability to react normally to the transferred signal molecules (Staehlin and Hull, 1978). Our observations also show that solitary gap junctions and gap junctions in association with tight junctions are greatly increased in the differentiated tumors.

Another role in which gap junctions have been implicated is nutritional, involving the exchange of small metabolites. In embryonic tissue (Potter et al., 1966) metabolic coupling appears to be vital for the distribution of nutrients before a circulatory system is established. In the adult organism certain avascular tissues (i.e. lens, Goodenough, Paul, and Culbert, 1978) are thought to be maintained by a similar exchange of metabolites. This may explain, in part, how BCC nodules, without an intranodular blood supply, can grow larger without extensive necrosis, centrally located cells being able to draw essential substances from cells positioned near the perphery. This is in contrast to the fairly frequent areas of necrosis that occur in many other neoplastic growths, as the result of precarious blood supplies. In connection with this aspect of the role of gap junctions, it will be interesting to see whether those few BCC which show central nodular necrosis contain less or no gap junctions. The random pattern of cell death that has been reported in BCC may reflect a genetically determined phenomenon (Kerr and Searle, 1972; Laird, 1969) rather than an alteration of the gap junctions.

The tight junctions which were described in both differentiated and undifferentiated tumor samples in our study were simple tight junctions usually consisting of a single, short strand. Tight junctions, as well as gap junctions were found on cells throughout the tumor nodule and were very similar to the focal tight junctions that have been described previously as maculae or fasciae occludentes (McNutt and Weinstein, 1973). There is the possibility that these focal tight junctions are a partial reflection of differentiation in BCC. Although there is still argument about the histogenesis of BCC (Kint, 1970; Lapis, 1976), most of the recent data are not contradictory to the original hypothesis of Krompecher (Krompecher, 1903) that these tumors originate from the pluripotent, undifferentiated cells of the basal layer of the epidermis or the cutaneous appendages. Tight junctions in normal human skin appear when the basal kerati-

nocytes differentiate into the cells of the granular layer (Caputo and Peluchetti, 1977). Again our semiquantitative data show more junctional foci in the differentiated tumors compared to the undifferentiated one. Further studies on other types of BCC showing different degrees of differentiation will be necessary to show a possible connection between the differentiation of this tumor and the appearances of junctional structures.

A recent study on carcinogen-induced rat urinary bladder carcinomas (Pauli et al., 1978) shows that the percentage of cell surface area occupied by desmosomes was greater in carcinomas than in controls. It was interpreted that a decreased intercellular adhesion is not a necessary prerequisite for tumor invasion. In human BCC desmosomes are generally numerous but in addition many other adhesive structures (gap and tight junctions) are present. Indeed in the differentiated samples *all* exposed membrane interfaces contained junctional foci. The contribution of these additional adhesive structures to the low invasive character of BCC cannot be ruled out at this time.

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