

## Cell surface alterations in embryonic tissues exposed to RGD-peptides: selective expression

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**ABSTRACT** Whole animal studies have implicated cell adhesion molecules in a diverse array of developmental processes. The present study reports on the morphological effects of RGD-related peptides on the cell surface of various living chick embryonic tissues. We report a novel and characteristic plasma membrane reaction that is caused by treatment with different RGD-peptides. Not only does each peptide evoke a response in certain tissues and not in others, but each brings about a specific type of plasma membrane reaction (bleb). Although the mechanisms are unknown, the specificity of this phenomenon suggests that it could provide a window into new surface interactions in morphogenetic systems.

KEY WORDS: *cell surface, RGD-peptides, embryonic tissues*

### Introduction

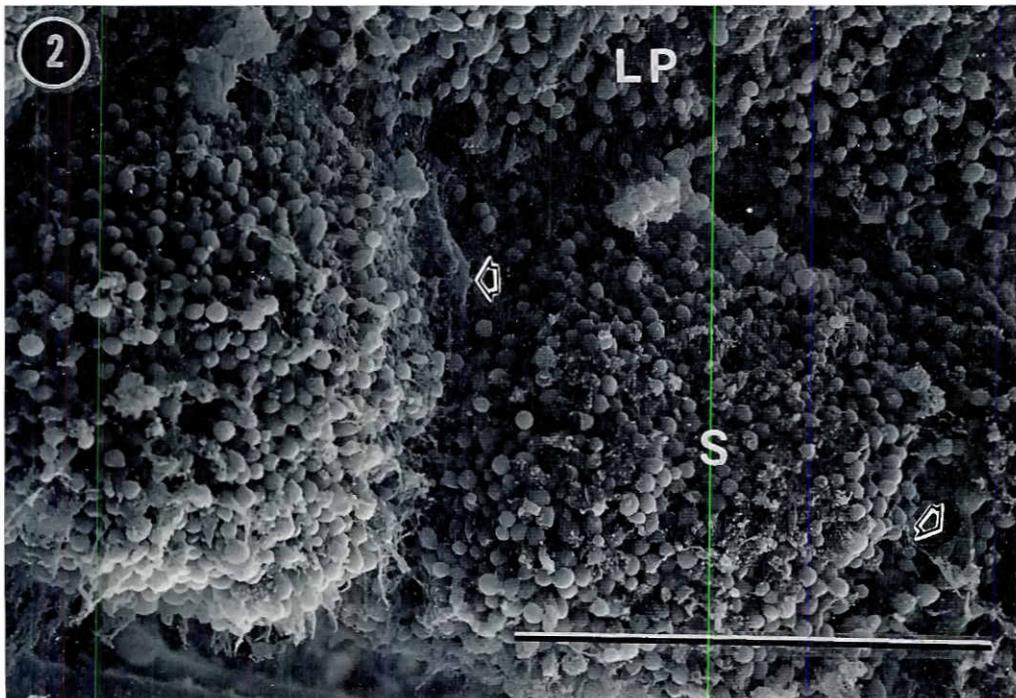
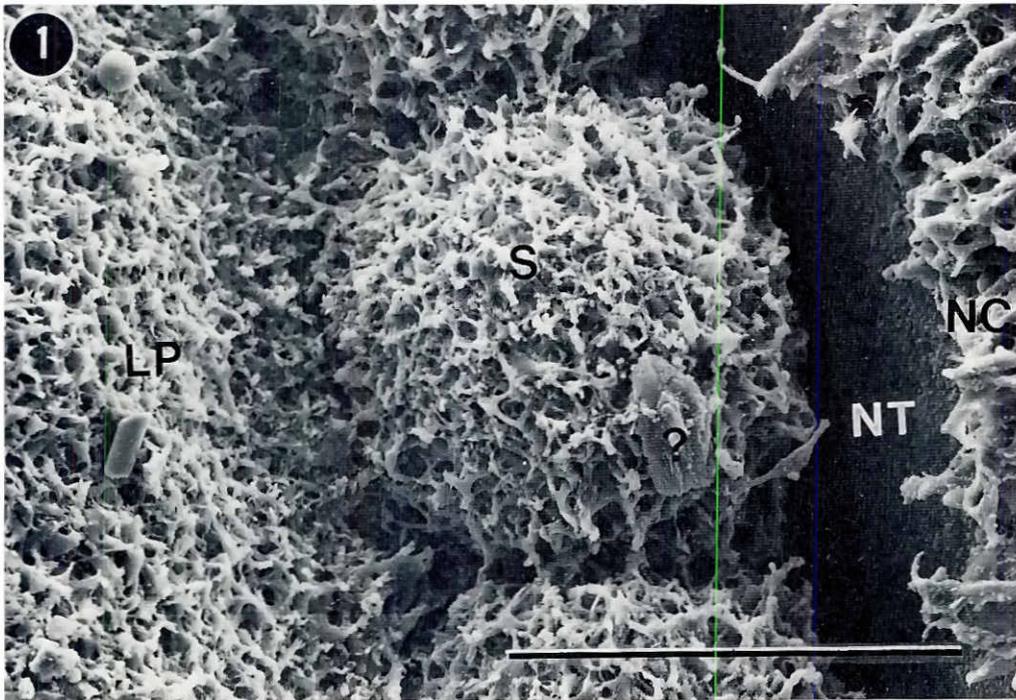
Cell adhesion molecules (CAMs) play an important role in embryonic development. Whole animal studies with homotypic CAMs, such as the cadherins and N-CAMs, have yielded significant information on such embryonic processes as gastrulation and neurulation (Takeichi, 1988), neural crest differentiation (Thiery *et al.*, 1990), myotube formation (Fredette *et al.*, 1993), axonal path finding (Tang *et al.*, 1992) and nephric duct extension (Bellairs *et al.*, 1995).

Whole animal studies have also implicated heterotypic CAMs such as integrins in a diverse array of developmental processes. The chick blastoderm has been found by Lash *et al.* (1990) to undergo important morphological changes when treated with RGD-peptides at Hamburger and Hamilton (1951) stages 4-5. Certain tailbud mesenchymal cells in older embryos have also been shown to undergo morphological changes in the presence of RGD-peptides (Mills *et al.*, 1990, HH stages 20-24). Drake and Little (1991) and Drake *et al.* (1992) have demonstrated  $\beta 1$  integrin-mediated adhesive changes in somitic mesenchyme in younger embryos (HH stages 15-17). SEM studies by Bellairs *et al.* (1995), also on RGD-peptide-treated younger embryos, have shown an array of cell surface alterations (blebs) in some, but not all, mesenchymal cells. The following developmental processes were also found to be perturbed by RGD-peptides, although cell surface alterations were not examined: neural crest migration and gastrulation (Boucaut *et al.*, 1984), somite formation (Lash *et al.*, 1984) and precardiac cell migration (Linask and Lash, 1986, 1988).

The integrin family of receptors, which interact with either fibronectin or a variety of other matrix or cell surface ligands, is becoming increasingly better understood, despite its great complexity (e.g. see recent reviews by Hynes, 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993; Clark and Brugge, 1995). Experiments, especially those involving the use of synthetic peptides that mimic key sites in fibronectin (reviewed in Yamada, 1991; Yamada and Miyamoto, 1995), have corroborated the notion that the integrin receptors and their specific ligands play a significant role in early development and morphogenetic movements. Little is known however about the effects of these specific ligands on embryonic cell surface morphology.

Of particular importance are the synthetic peptides containing the RGD (arginine-glycine-aspartic acid) sequence of fibronectin, which can function as competitive inhibitors of cell adhesion and migratory processes *in vitro* and *in vivo* (Ruoslahti and Pierschbacher, 1987; Yamada, 1991). Although some variants of RGD peptides are inactive in certain biological assays, they can display unanticipated activity in other assays for cell adhesion, suggesting complex cell surface recognition events beyond simple binding to RGD. For example, soluble RGD variants containing RGE or DGR (reversed sequence) display specific inhibitory activity in certain adhesion and ligand-binding assays (Humphries *et al.*, 1986; Yamada and Kennedy, 1987; Mould *et al.*, 1990). Soluble RGD-containing peptides can also directly promote a biological activity: occupancy of integrin receptors by RGD peptides can mimic part of the activity of intact fibronectin by inducing receptor redistribution to focal adhesions (LaFlamme *et al.*, 1992), as well as synergizing with integrin

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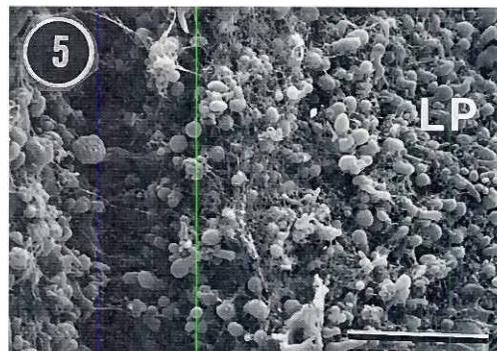
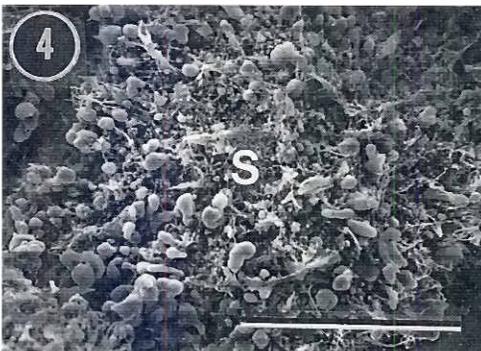
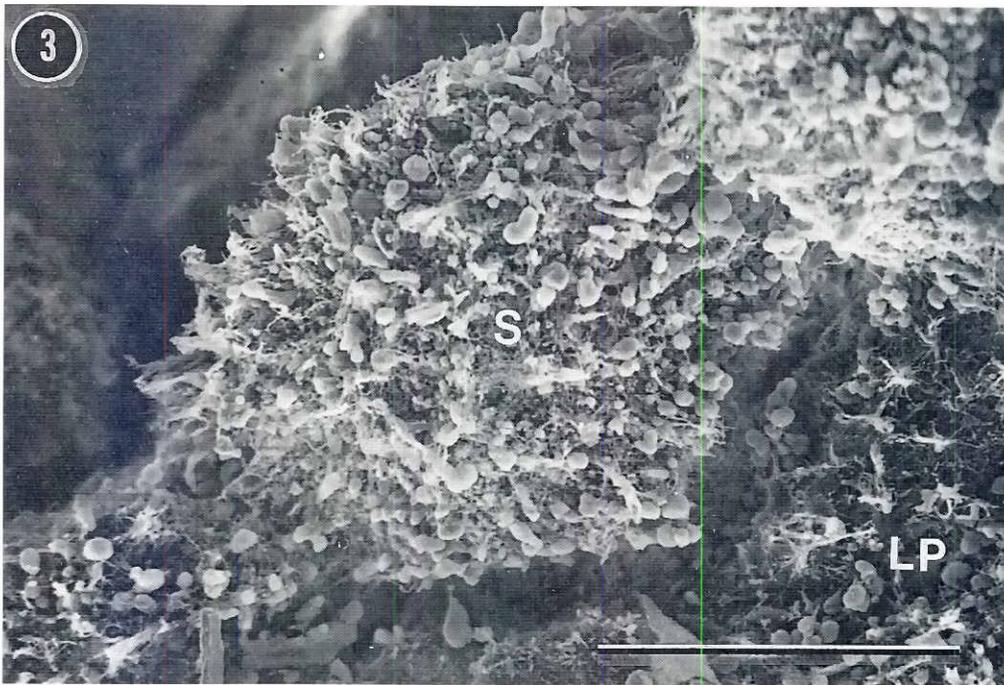
**Fig. 1.** Scanning electron micrograph (SEM) showing the typical stellate appearance of lateral plate, somite, and neural crest cells in a HH stage 17 control embryo. LP, lateral plate; S, somite; NT, neural tube; NC, neural crest. (? , contaminating particle) Bar, 100  $\mu$ m.

**Fig. 2.** SEM showing blebs of relatively uniform size in the most posterior 2 somites of a HH stage 18 embryo after treatment with GRGDS. S, last formed somite; LP, lateral plate. Arrows indicate two rare unperturbed cells of unknown cell type. Bar, 100  $\mu$ m.

receptor aggregation to induce cytoskeletal protein aggregation (Miyamoto *et al.*, 1995).

The aim of the present study was to identify other activities of these RGD-related peptides by testing their effects on various living tissues. Of the many RGD-containing molecules, we also tested vitronectin. This protein was chosen because it is a naturally occurring RGD ligand that interacts with at least four wide-

ly distributed RGD-binding members of the integrin receptor family (Felding-Habermann and Cheresch, 1993). We report here on a novel cell surface interaction that is caused by treatment with different RGD-peptides. Although its mechanisms are unknown, the specificity of this phenomenon suggests that it could provide a window into new surface interactions in morphogenetic systems.



**Fig. 3.** SEM of HH stage 13 embryo treated with SDGR showing blebs of irregular size and shape on a somite (S). LP, lateral plate. Bar, 50  $\mu$ m.

**Fig. 4.** SEM of somites and after treatment with GRGDS. Cell surface alteration is not as extreme as with GRGDS treatment. S, somite. Bar, 50  $\mu$ m.

**Fig. 5.** SEM of lateral plate mesoderm (LP) of the embryo shown in Figure 3. S, somite. Bar, 20  $\mu$ m.

## Results

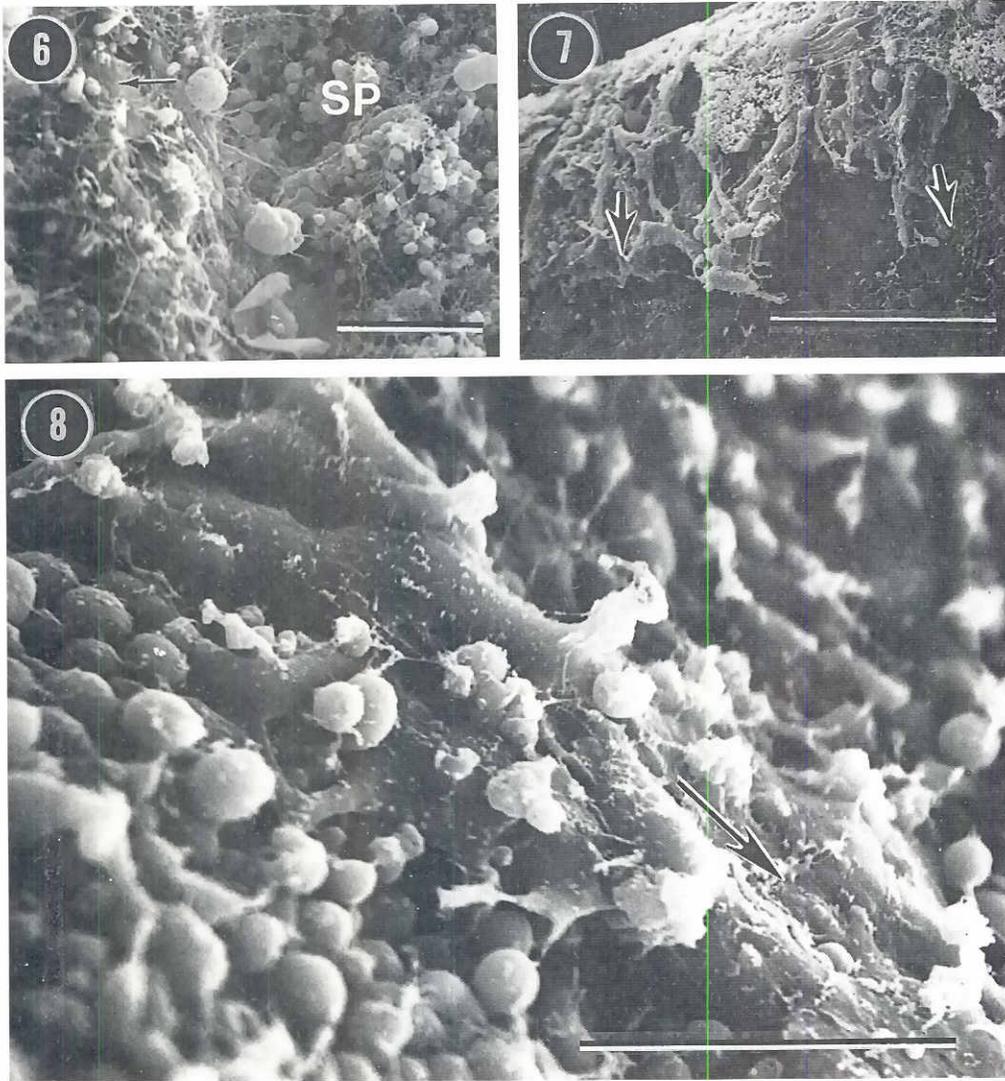
The normal mesenchymal appearance of SEM-observed embryonic avian mesoderm is shown in Figure 1. Cell processes and extracellular matrix are prominent in this preparation. Cell surface alterations have been seen in the following tissues after treatment with RGD-ligands at stages between HH4 and HH24: somites and lateral plate mesoderm (Fig. 2), somite and lateral plate mesoderm (Fig. 3), and somite mesoderm (Fig. 4). In Figure 2, rare but obvious unperturbed cells can also be seen, although their identification is not known. Blastoderm cells in the presence of GRGDS retract their cell processes and although they do not form blebs, they alter their surface morphology (cf. Fig. 4c in Lash *et al.*, 1990). Cell surface alterations or blebs have not been seen in neural or ectodermal tissues, nor in the endoderm.

The ability of a tissue to react to a specific peptide can change as that tissue matures. For example, the anterior region of the segmental plate shows a greater response to GRGES than the posterior region (cf. Bellairs *et al.*, 1995). The ability to react

varies also according to the tissue. The tailbud somite cells show a strong response to GRGDS whilst the neighboring lateral plate does not exhibit any alteration in cell surface morphology (cf. Fig. 18 in Mills *et al.*, 1990).

The size and shape of the blebs are not always uniform, and they vary according to the peptide used (Table 1). Blebs produced in response to GRGDS (cf. Fig. 2) are more regular in size and shape than those due to SDGR, which are highly irregular (Fig. 3). Similar irregular blebs and processes are also observed after treatment with GRGES (not shown), whilst irregular blebs and thicker processes are characteristic of the response to vitronectin (Fig. 6).

A striking specificity of response is seen in some instances. In the tailbud, perturbed unsegmented paraxial mesoderm is sharply delineated from closely apposed and unaffected lateral mesoderm (not shown here, cf. Fig. 4 in Mills *et al.*, 1990). Neural crest cells without blebs continue their migration (Fig. 7) over a normal-appearing neural tube (cf. Bancroft and Bellairs, 1976) in a GRGDS-treated embryo. In this same embryo, somite cells exhibit characteristic GRGDS-stimulated blebs (cf. Fig. 2).



**Fig. 6.** SEM showing the effect of vitronectin on the mesenchymal cells. Whereas the mesenchymal cells produce many blebs of irregular size, the nephric duct contains far fewer blebs. The arrow points to the smooth surface of the duct mesenchyme beneath the granular and fibrous extracellular matrix. SP, segmental plate. Bar, 20  $\mu$ m.

**Fig. 7.** SEM showing neural crest cells migrating over the surface of the neural tube and adjacent mesenchyme after GRGDS treatment. The surface of the neural tube is unperturbed by the peptide treatment whereas the surrounding mesenchyme has produced blebs (not readily seen here) similar to those shown in Figure 2. Arrows indicate direction of neural crest migration. Bar, 50  $\mu$ m.

**Fig. 8.** SEM showing the nephric duct extending posteriorly over a cellular substratum with cellular blebs induced by GRGDS treatment. The arrow indicates the direction of duct extension. Bar, 20  $\mu$ m.

And finally, the nephric duct (Fig. 8) appears unperturbed as it continues its extension over a substratum exhibiting blebs.

### Discussion

This study identifies distinctive effects on the cell surface architecture of certain living embryonic avian tissues by specific peptides related to the RGD sequence. The extent and pattern of blebbing displays specificity that depends on the amino acid sequence of the peptides.

When embryos were treated with RGD peptides the surfaces of certain tissues underwent blebbing. Each peptide produced its own characteristic type of blebbing pattern (Table 1). For example, treatment with GRGDS produced blebs regular in shape and of uniform size, typically illustrated in somite cells in Figure 2. By contrast, treatment with SDGR caused blebs of irregular shape and size, typically illustrated in Figure 4. Similarly, both GRGES and vitronectin each produced their characteristic type of irregular blebs and processes (Figs. 3 and 6). Blebs have been report-

ed previously in untreated cells in culture (Erickson and Trinkaus, 1976; Yamada *et al.*, 1976) and are thus not restricted to cells treated with peptides.

The biochemical basis of such blebbing is uncertain, though a general feature is that it is not conducive to cell or tissue migration. The production of blebs is generally considered to result from loss of contact with neighboring cells or with adjacent extracellular matrix. Contacts with a substratum are of especial importance in developing tissues and are maintained by elongated lamellae and filopodia. It seems probable that the blebbing reported in this paper results from loss of contact between cell membrane and substratum.

Blebbing is also a feature of early mitosis and can be seen in time-lapse studies of cells which have lost these contacts and rounded up preparatory to mitotic division (Trinkaus, 1984) but it is not likely that the peptide-induced blebbing reported here is a prelude to mitosis since the number of cells affected is too large, and upon recovery (Lash *et al.*, 1990) the tissue looks normal without any apparent increase in size.

TABLE 1

## BLEBBING RESPONSE OF DIFFERENT TISSUES

Treatment	Somites	Seg Plate	Neph Duct	Lat Plate	Bleb size
Controls	-	-	-	-	None
GRGDS	+	+	-	+	Regular
SDGR	+	+	-	+	Irregular
GRDGS	-	-	-	+	Regular
GRGES	+	+	-	+	Irregular
Vitronectin	+	+	+	+	Irregular

In some instances the blebs are of uniform size and shape (regular), whereas in other instances the blebs are variable in size and shape (irregular). Blebs present (+). Blebs not present (-). Tissues examined: somites, segmental plate, nephric duct, and lateral plate. Neural crest cells were examined only with GRGDS treatment (cf. Fig. 7).

Drake and Little (1991) and Drake *et al.* (1992) have convincingly demonstrated that the RGD-peptide produces dramatic changes in the relationship between chick somites and the surrounding tissues. Whereas their observations did not permit the detection of blebs, the somites clearly showed a loss of contact with the neural tube and surrounding tissues. This is a good example of one integrin-mediated adhesive change in somitic tissue.

Blebbing can be seen in time-lapse studies of cells which have lost these contacts and rounded up preparatory to mitotic division (Trinkaus, 1984) and this type of blebbing is probably due to a redistribution of cell membrane (Harris, 1973). It seems probable therefore that the blebbing reported in this paper also results from loss of contact between cell membrane and its substratum.

The cells at the periphery of the young chick blastoderm are attached to their specific substratum, the inner surface of the vitelline membrane, by long lamellae which enable them to migrate (Bellairs *et al.*, 1969; Andries and Vakaet, 1985). Treatment with GRGDS-peptides results in loss of these attachment lamellae (Lash *et al.*, 1990) whilst recovery involves their reattachment and the resumption of migration. Recovery in these experiments occurred within 2 h (Lash *et al.*, 1990). Whereas a detailed study of recovery could not be performed in this present study, cessation of extension was still evident in some embryos after 4 h. The culture conditions did not permit longer periods of observation.

Additional support for the existence of specific cell surface receptor responses to peptides potentially related to those reported here can be derived from the report of Miyamoto *et al.* (1995), where complex ligand-receptor effects were demonstrated on intracellular components associated with cell movement and attachment. Not all migrating tissues, however, appear to be dependent on fibronectin-mediated contacts. The nephric duct cells continue to migrate after treatment with RGD-peptides and the duct extends over the lateral plate (its normal substratum), even though the latter tissue contains highly blebbed cells (Bellairs *et al.*, 1995).

The present account illustrates characteristic plasma membrane reactions caused by treatment with different peptides. Not only does each peptide evoke a response in certain tissues and not in others, but each brings about a specific type of bleb.

Blebbing of these embryonic cells differs according to circumstances. The evidence comes not only from our findings that a tissue may respond differently to different peptides, but also from the fact that a tissue which does not respond to a particular peptide at one stage of differentiation may do so at a later stage. The anterior end of the segmental plate exhibited blebbing with GRGES at 200-250 µg/ml, but the posterior end of the segmental plate did not respond at all unless the concentration was increased to 500 µg/ml (cf. Bellairs *et al.*, 1995). A similar difference in response of the anterior and posterior regions of the segmental plate was found after treatment with SDGR (Bellairs *et al.*, 1995). There was an apparent difference in the response of more mature regions of this tissue. The cells that were about to become somites (i.e. the anterior segmental plate) responded as did the somites. By contrast, segmental plate tissue that had recently formed after gastrulation, had not yet acquired this response mechanism.

GRGDS treatment led to blebbing in the lateral plate mesoderm of the trunk, but not that of the tail, which is in a less mature state (Mills *et al.*, 1990). This situation was similar to that reported above in the segmental plate.

A striking example of specificity can be seen in Figure 2. A few normal looking cells, which may be neural crest cells, can be seen in the midst of the blebbed cells. Although their exact identity is unknown, they clearly show a marked difference from their neighbors in their response to the peptides.

During the differentiation of embryonic tissues dramatic changes occur at the surfaces of their cells. The surface alterations of embryonic cells as a response to the RGD-peptides is a demonstration of how vital cell contacts are, and how sensitive the cells are to perturbations with peptides, analogs related to the RGD-sequence of the fibronectin molecule, and to another RGD-containing cell adhesion molecule, vitronectin.

## Materials and Methods

Hens' eggs were incubated to HH stages 9-24 (Hamburger and Hamilton, 1951). The embryos were removed from the yolk and rinsed in phosphate buffered saline (PBS) at pH 7.4, and transferred to 35 mm plastic dishes for staging and trimming of excess extra-embryonic membranes.

Synthetic peptides were purified on Bio-Gel P2 columns (Bio-Rad, Richmond, CA, USA), reversed phase HPLC using a preparative C18 column, and then passed through a Dowex-1 column. The RGD-peptides (and their analogs) used were: GRGDS, GRDGS, SDGR, and GRGES. The purity of the peptides was confirmed by analytical reversed phase HPLC. An RGD-containing ligand, vitronectin, was also tested.

Forty-nine embryos of HH 12-14 were removed from the yolk and dissected free of extra-embryonic membranes. Four were fixed immediately as controls (i.e., untreated embryos). In the remaining specimens, the anterior part of the body was removed by a transverse cut at the level of the heart and discarded. The individual trunks were immersed in separate multiwells (Sterilin, Teddington, UK) with 0.5 ml of culture medium, containing no additives (10 incubated control specimens), or containing either vitronectin (3 specimens) or one of the following peptides; GRGDS, GRDGS, SDGR, GRGES (6 to 9 specimens per peptide). The culture medium consisted of Earle's 199 (Wellcome), fetal calf serum and penicillin-streptomycin (Gibco) in the ratio of 9:1:0.5 by volume. The vitronectin was used at a concentration of 200 µg/ml, whilst the peptides were at 200-250 µg/ml (or 500 µg/ml in 4 of the 8 specimens treated with

GRGES). The specimens were incubated for 2 h in an atmosphere of air containing 5% CO<sub>2</sub>.

For scanning electron microscopy the specimens were washed 3 times with PBS and fixed overnight in 2.5% glutaraldehyde in 0.1 M PBS at pH 7.4. After further rinsing in PBS they were treated for 1 h with 1% osmium tetroxide in PBS. The ectoderm was dissected from the specimens which were then critical point-dried from liquid carbon dioxide. The tissues were then dehydrated in graded acetones, affixed with carbon paint onto stubs, gold coated and examined in a Hitachi S-530 scanning electron microscope.

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