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Scientific Section

REVIEW SERIES

Signal Transduction

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Abstract. *This review outlines some of the developments in intra cellular cell signalling. Details are presented on how growth factors stimulate cells to respond as well as interactions between extracellular matrix and cell adhesion molecules. The relevance of these activations to orthodontics offers scope for further research.*

Index Words: Growth Factors, Cell Adhesion Molecules, Second Messengers

Introduction

One of the fastest moving areas in biological sciences is understanding the mechanisms by which hormones or ligands stimulate reactions within cells. The purpose of this review is not to reintroduce basic concepts of cell signalling which have been reviewed previously in clinical orthodontic journals (Sandy and Farndale, 1991; Sandy and Farndale, 1993) but to provide an update on some of the most exciting discoveries since the previous reviews were written. It is also hoped that where possible the relevance to some of the biological reactions in craniofacial development and orthodontics will be referred to.

In it's simplest form, signal transduction can occur with receptors that essentially constitute channels. When these receptors are activated by interacting with a stimulus, signals in the form of ion movement in or out of the cell produce changes in electrical potential which then enables a signal to be propagated. More sophisticated signal transduction pathways usually involve a ligand-receptor-complex which activates intracellular events. The culmination of these activations is a change of cellular activity often resulting in protein formation or changes in the expression of genes within the activated cell.

One of the most difficult concepts to grasp is that activation of a signal pathway in one cell type may produce a completely different reaction in another cell. As more is understood about mechanisms for modulation of signal pathways it becomes increasingly clear that subtle modifications can be produced in receptors by single-transmembrane-domain proteins known as receptor-activity-modifying proteins or RAMPs. These are relatively recently described (McLatchie et al., 1998) and are capable of modifying the behaviour of a calcitonin receptor so that it functions either as a calcitonin gene related peptide (CGRP) receptor or an adrenomedullin receptor. These switches have a significant effects on the different reactions from the receptors. CGRP appears to be a modulator of the effects of orthodontic force on alveolar bone osteoblasts and osteoblast like cells. It is thought that CGRP may initiate osteoblasts activity and therefore RAMPs may have some role in initiating this process (Saito *et al.*, 1996). This novel concept offers a model for altering receptor behaviour which will then trigger a different set of intracellular events.

The guanine nucleotide binding (G) proteins relay signals from transmembrane receptors to intracellular enzymes and ion channels, mediating reactions such as vision, smell, taste and the actions of many neurotransmitters and hormones. Recent developments have suggested that mutations in these G-proteins will modify signal activation and this provides a new area for investigating disease mechanisms (Liri *et al.*, 1998). Discoveries such as these, coupled with cell imaging and molecular biology advances have provided considerable impetus in this field.

The complexity of cell signalling is further modified by the physical linkage between cytoskeletal structures and the extracellular matrix (ECM). An example is the family of integrins and cadherins which form transmembrane links to the ECM. The integrins and cadherins can modulate signals from growth factors, as well as generating signals during interaction with the ECM. The cellular events controlled by this signalling path includes motility, differentiation, cell division and programmed cell death (Gumbiner, 1996). They are therefore likely to be implicated in many events controlling tissue morphogenesis as well as tumour progression (Howe *et al.*, 1998). The mechanisms for controlling cell death have become areas of considerable interest in both developmental biology and cancer studies. It seems that far from being a random event, cell death is essential for many developmental processes.

Guanine nucleotide binding proteins have been reviewed elsewhere (Sandy and Farndale, 1991). Receptors which couple to G-Proteins all have a similar structure which is characterised by seven transmembrane spanning domains. G proteins interact directly with the receptor and in this coupled state, G proteins are known to control a number of events. These include stimulation of Na^+/H^+ exchange, Ca^{2+} influx, and activation of a number of enzyme systems which act as second messengers. Specific

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forms of phospholipase C hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) to yield inositol 1,4,5,-trisphosphate (InsP3) which can stimulate release of Ca^{2+} from intracellular stores. Diacylglcerol is also produced which activates protein kinase C. Other enzyme systems which are known to be affected by activated G proteins include adenylate cyclase and phospholipase A2 which hydrolyzes phospholipids to produce arachidonic acid. The latter provides a substrate for the production of eicosanoids by lipoxygenase and cyclo-oxygenase activity. Adenylate cyclase generates cyclic AMP (cAMP) from ATP and the regulation of this enzyme by G proteins is well known (for reviews see Crouch and Hendry, 1993, Spiegel, Shenker and Weinstein, 1992).

Tyrosine Kinase Receptors

These receptors have intrinsic enzymatic activities which include tryosine kinases and tyrosine phosphatases. The proteins which encode receptor tryrosine kinases have four major portions.

- an extracellular domain for binding of growth factors or ligands
- an intracellular domain for activation of tryrosine kinases
- an intracellular domain which regulates activity
- a transmembrane domain which is thought to have anchoring properties.

The receptor tryrosine kinase proteins are classified into groups depending on structural features of their extracellular portions.

The highly conserved cytoplasmic tyrosine kinase domain will autophosphorylate and in this state act as a binding site for proteins with *src* homology 2 (SH2) domains (Ullrich and Schlessinger, 1990). These include phospholipase Cγ (PLCγ) phosphatidylinositol 3-kinase (PI3-kinase) and GTPase activating protein of p21 ras (GAP). The association of molecules containing SH2 domains with the receptor may lead to phosphorylation and activation. This appears to be true for PLCγ (Nishibe *et al.*, 1990) and PI3-kinase (Giorgetti *et al.*, 1993).

Mitogen Activated Protein Kinases (MAPK)

Mitogen activated protein kinases were initially identified in cells which had been stimulated with growth factors. They were therefore labelled as mitogen activated protein kinases. The MAP kinases were thought to respond specifically to growth factors but other stimuli such as phorbol esters (which function by activating protein kinase C) or bombesin which functions via G-proteins as well as electrical stimulation can cause tryrosine phosphorylation of MAP kinases. The MAP kinases operate as a cascade with MAP kinase kinase kinases activating MAP kinase kinase which in turn activates MAP kinase. Translocation of MAP kinases to the nucleus offers a means to control transcriptional regulators such as the proto-oncogenes *fos, myc* and *jun* (Figure 1).

Growth Factor Signal Pathways

A good example of how growth factors stimulate signal pathways is to examine the events associated with the growth factor platelet derived growth factor (PDGF).

Two distinct PDGF receptor types have been identified, the α receptor which binds all three isoforms (PDGF-AA,

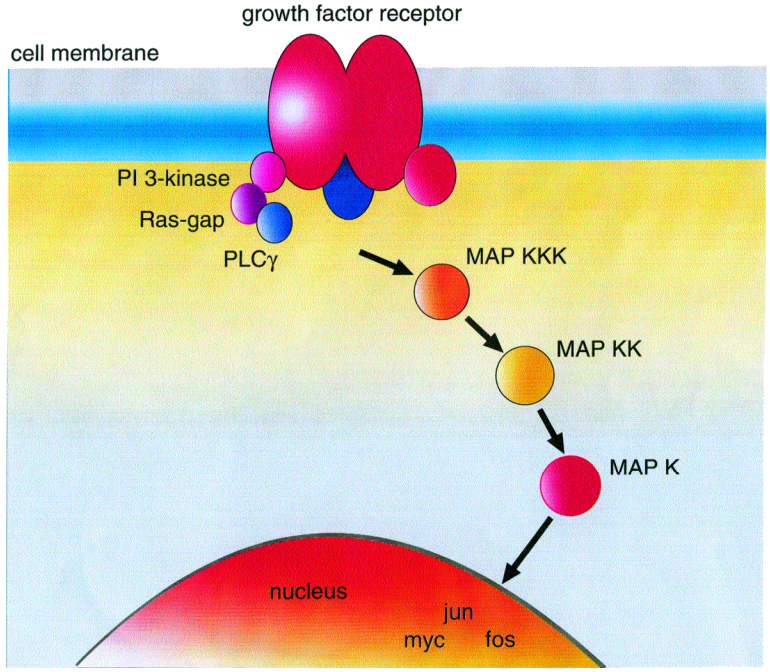

FIG. 1 Scheme to show growth factor receptor activation and it's effect on the MAP kinase cascade. When a growth factor binds to the receptor, the receptor autophosphorylates and in this state is a binding site for a number of proteins (PI3 Kinase, RAS GAP and PLC). These have their own activities but the tyrosine kinase receptors and indeed the non-tyrosine kinase receptors can activate the MAP cascade. The eventual translocation of MAP kinase to the nucleus controls transcriptional regulators such as the proto-oncognese fos, myc and jun.

PDGF-AB, PDGF-BB) and the β receptor which only binds PDGF-BB. The two types of receptor are similar in structure with an extracellular ligand-binding portion, a single transmembrane anchoring domain and a highly conserved intracellular protein-tyrosine kinase. When PDGF binds to the extracellular portion, the receptor undergoes dimerization and autophosphorylation with activation of the tyrosine kinases. The ligand receptor complex is then internalized and degraded. The secondary responses to the autophosphorylated receptor include:

a) Activation of phospholipase A_2 with release of arachidonic acid, which via cyclo-oxygenase and lipoxygenase activity, leads to the formation of prostaglandins and leukotrienes. The production of prostaglandin E_2, through an autocrine action is known to elevate cAMP levels in bone cells and this is usually associated with an inhibition of mitogenesis (Farndale *et al.*, 1988; Yamaguchi *et al.*, 1988). Increasing prostaglandin levels may therefore act as an amplification loop for growth factors since prostaglandins are produced as a by product which will then stimulate the cell further.

b) Activation of phospholipase C-γ through a G protein with degradation of PIP2 with formation of IP3 and DAG.

   Two intracellular processes are activated by this route. First calcium is released from endoplasmic reticulum stores by IP3 and second protein kinase C (PK-C) is translocated into the membrane with signal transduction via serine-threonine kinase activity. There is also good evidence that prostaglandins activate inositol phosphates (Farndale *et al.*, 1988) and since PDGF activates phospholipase A_2 activity the inositol phosphate pathway is of significance in the mediation of PDGF effects.

c) Recruitment of substrate proteins to an oligomerized growth factor receptor with increased tyrosine kinase activity.

   This includes, PI 3-kinase, Ras-GAP and PLCγ. Several other proteins are substrates for tyrosine kinases and amongst these are the MAP kinases. Two of these forms, 42 and 45 kDa, are tyrosine phosphorylated and activated in response to PDGF (Cooper *et al*., 1982; Rossomando *et al*., 1989). Whilst they are unlikely to be direct substrates for the PDGF receptor, the activation of this cascade together with evidence that growth factors induce nuclear transfer of MAP kinases (Lenarmond *et al.*, 1993) suggest this pathway is important in mediating growth factor responses (Figure 2). Perhaps the most compelling evidence that this pathway is important in mitogenesis in bone cells comes from the observation that inhibitors of tyrosine kinases can block PDGF stimulated cell proliferation and receptor phosphorylation (Davidai *et al*., 1992; Sandy *et al*., 1998). Figure 3 shows the detection of the phosphorylated PDGF receptor with tryrosine kinase activity.

Insulin like Growth Factors

Insulin like growth factors (IGF-I and IGF-II) are also of relevance. The IGF-I receptor (type 1) is structurally

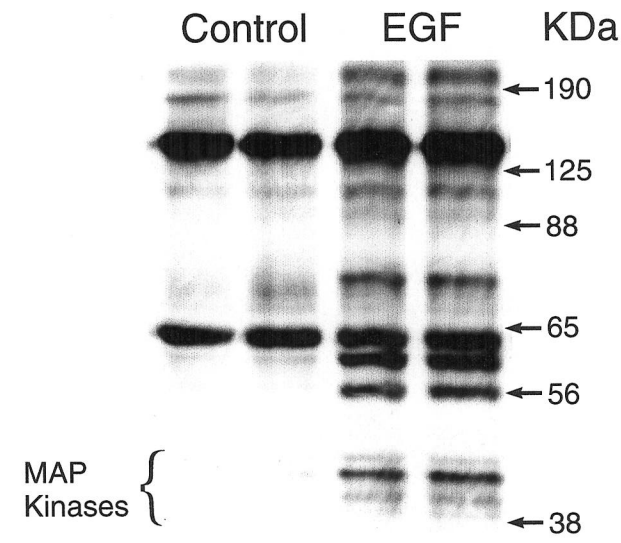

FIG. 2 Cultures of bone cells were exposed to epidermal growth factor (EGF) 4ng/ml for 5 min. The reaction was terminated by the addition of hot sample buffer and proteins immediately separated by SDS-PAGE. After western blotting, the proteins were analysed with a specific antiphosphotyrosine antibody (anti -*P*Y, polyclonal, 1:5000). The two control lanes can be compared with the two EGF stimulated lanes. The EGF's receptor is identified by the band at the 190kDa marker. The stimulation of the MAP kinases can be seen as three distinct bands above the 38kDa marker. These bands do not exist in the control lanes and are clearly stimulated by EGF.

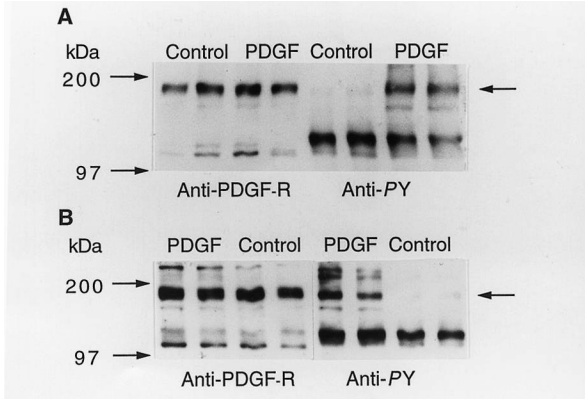

FIG. 3 Cultures of bone cells were exposed to PDGF (8ng/ml) for 5 min. The reaction was terminated by the addition of hot sample buffer and proteins immediately separated by SDS-PAGE.
A shows identification of PDGF receptor as definitive phosphorylation site. After western blotting, the membrane was cut in half and in one portion the proteins were analysed with a specific antiphosphotyrosine antibody (anti-*P*Y, polyclonal, 1:5000). An antibody to the PDGF receptor (anti-PDGF-R, polyclonal, 1:1000) was used to examine proteins in the second portion. The PDGF receptor antibody identified a band in both controls and PDGF stimulated cells at about 170kDa. The phosphorylation of this band was only seen in cells stimulated with PDGF.
B Identification of the PDGF receptor as phosphorylation site when stimulated with PDGF. After Western Blotting, the proteins were analysed initially with anti-PDGF-R (1:1000) and immunodetected. The blot was then stripped and the proteins re-analysed with a specific anti-*P*Y (1:5000). With anti-PDGF-R, a band corresponding to the receptor weight (180kDa) was detected in both the controls and PDGF stimulated cells. When the blot was stripped and probed with anti-*P*Y, only cells which had been stimulated with PDGF demonstrated phosphorylation of this same band.

similar to the insulin receptor, a transmembrane glycoprotein with an extracellular ligand-binding domain and a cytoplasmic portion with tyrosine kinase activity. The type I receptor binds IGF-I most avidly but can also bind insulin and IGF-II (Cohick and Clemmons, 1993). As with the PDGF receptor, the IGF-I receptor appears to mediate IGF-I actions by phosphorylating cellular substrates. The insulin receptor substrate (IRS-I) acts as a multi site 'docking' protein for cellular proteins such as PI3-kinase. The use of kinase deficient IGF-I receptors and IGF-I antibodies has suggested that tyrosine kinase activity is necessary for activation of IGF-II stimulated signal transduction cascades (Kato *et al.*, 1993).

The IGF-II receptor is identical to the cation independent mannose-6-phosphate receptor which functions as a lysosomal enzyme targeting protein (Cohick and Clemmons, 1993). The IGF-II receptor consists of a single polypeptide chain with a large extra cellular domain, a single transmembrane portion and a short cytoplasmic region which lacks intrinsic protein kinase activity. The type 2 receptor preferentially binds IGF-II to IGF-I (Scott and Baxter, 1987). It has recently become apparent that IGF-II also relays through the IGF-I receptor and sometimes through the insulin receptor. In fibroblasts, the effects of IGF-II on both Ca^{++} influx and DNA synthesis are mediated through the IGF-II receptor. This receptor appears to link two signal transduction pathways through a pertussis toxin sensitive G Protein (Nishimoto *et al.*, 1987) which appears to be Gi2α (Okamoto, 1991). This coupling of Gi2α is of significance because it demonstrates that receptor recognition of G Proteins does not necessarily require a 7 transmembrane structure (Murayama *et al.*, 1990, Okamoto *et al*., 1990). There is therefore evidence which suggests that the signal transduction of IGF-II may occur through G Proteins, the type 1 receptor which may in turn link to phosphoinositide hydrolysis and tryosine kinase activity. The result of activating several pathways simultaneously is therefore difficult to relate to a single cell function.

Transforming Growth Factor β Signalling

Transforming growth factor β 1 (TGFβ1) is one of a very large family of cytokines which includes the TGFβ, activins, inhibins and bone morphogenetic proteins. They have a considerable range of biological activities including cell growth, differentiation and apoptosis. They are involved in developmental processes and bone remodelling. The receptors for activins and TGFβ have intrinsic serine/threonine kinase activity and these pathways are different from receptors with intrinsic tyrosine kinase activity or activation of intracellular tyrosine kinases. This pathway is less well understood than the receptor tyrosine kinase pathways. Most recently (Heldin, *et al*., 1997) a family of signal transducer proteins (SMAD) have been identified and presents a mechanism by which TGFβ can signal from the cell membrane to the nucleus. The SMAD family are phosphorylated by cell surface receptors with serine/threonine kinase activity and in this state translocate to the nucleus where transcription factors produce the cells response to TGFβ. Furthermore, tyrosine kinase receptor activation of MAP kinases has been shown to phosphorylate SMAD proteins with inhibition of translocation to the nucleus (Kretzschmar *et al*., 1997). It is evident from this that the signal pathways have several mechanisms for cross communication.

Cell Adhesion Molecules and Signalling

Cells need to interact dynamically with adjacent cells and the extracellular matrix. The units of the cell adhesion system are broadly divided into three main classes.

1) Cell adhesion molecules (CAMs)
2) Extra cellular matrix proteins
3) The cytoplasmic linking proteins

The cytoskeleton is linked to the extracellular matrix through transmembrane glycoproteins (Figure 4). The

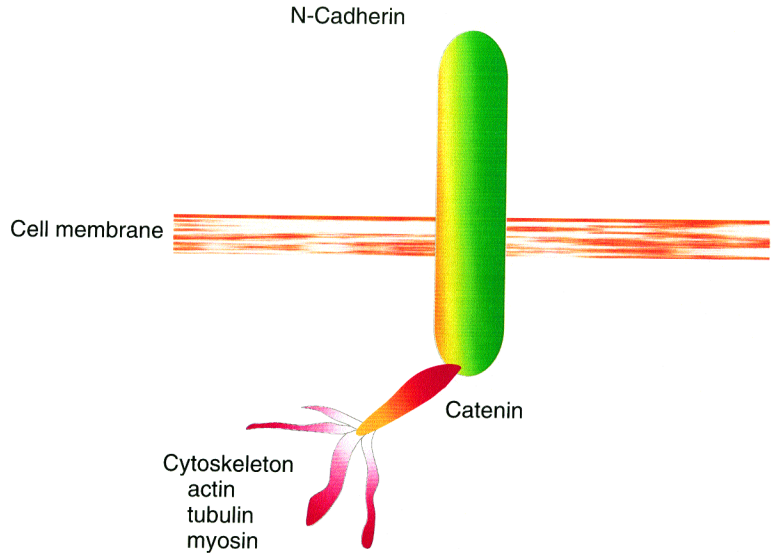

FIG. 4 Scheme to demonstrate how transmembrane glycoproteins such as cadherin or integrin are bound to the cytoskeleton. Clearly any distortion of the extracellular matrix (not shown) would have some influence on the transmembrane linking proteins and the cytoskeleton.

extracellular maxtrix proteins comprise; collagens, fibronectins, laminins and proteoglycans. They have the ability to bind to cell adhesion molecules and links between the extracellular matrix proteins and the cytoskeleton are provided by the transmembrane glycoproteins (for review see Kerrigan *et al.*, 1998).

The Integrins

The integrins bind to a wide variety of extracellular matrix molecules and other cell surface proteins. They are involved in cell matrix and cell-cell adhesion in many different biological processes. The integrins serve as transmembrane bridges between the extracellular matrix and the actin filaments of the cytoskeleton. It is now realised that the integrins and other adhesion molecules (selectins, immunoglobulin and cadherins) are involved in the signal transduction processes. Adhesion to extra cellular matrix proteins can activate the cytoplasmic tyrosine kinases (MAP kinases), serine/threonine kinases, induce ionic fluxes and activate the phosphoinositide signalling pathways. Integrin signalling can also activate receptor tyrosine kinases which are similar to those activated by growth factors. Indeed, the integrin regulation of growth factor signalling is now recognised as one of the most potentially interesting areas. Good evidence exists that the distribution and expression of integrin is upregulated when bone cells are mechanically distorted (Carvelho *et al*., 1995). Clearly during orthodontic tooth movement where extracellular matrix will be mechanically distorted, this may present a new potential mechanism for activation of signal cascades.

What new mechanisms for transduction of mechanical forces can be postulated?

The signal pathways utilised in mechanical perturbation of tissue are wide and varied. A large number of tissues are subjected to mechanical distortion in normal function (lung, muscle, cardiac, skeletal (Vandenburgh, 1992). The linkage of ECM to cytoskeleton must be involved in mechanical transduction processes, possibly directly to the nucleus. Alternatively, either indirectly (through growth factor production) or directly, the MAP kinases may play a significant role in nuclear response to mechanical forces. Further RAMPs are bound to be discovered and these may confer specificity for receptor responses. These may be modified under different conditions such as pressure or tension offering a new concept in explaining how these changes produce such different reactions.

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