CHAPTER 4
DISCUSSION

Dietary fatty acids have a profound role in the growth regulation of epithelial cells. The epidemiological and experimental evidence from the study of dietary components on diet, nutrition and cancer concluded that there is a causal relationship between dietary fat intake and incidence of cancers of the colon and breast (Tannenbaum 1942). Similar but less consistent correlations have been reported with cancers of the prostrate, ovary and endometrium (Armstrong and Doll 1975; Miller 1981). This is an attempt to investigate the role of dietary fat on lung epithelial cell proliferation and its role in the etiology of cancer. The transformation of a normal cell to a cancerous one is a complex process which can be induced by a variety of agents.

Studies from many laboratories have shown that polyunsaturated fatty acids (PUFA) are involved in the control of cell proliferation. Studies with some cells and tissues demonstrate stimulatory effects, whereas studies with other cells and tissues demonstrate inhibitory effects. Essential fatty acids (EFA) support growth, wound healing, and tumorigenesis and yet an EFA deficiency leads to hyperplasia.

4.1 ROLE OF DIETARY FATS IN NORMAL CELL FUNCTION AND CANCER PROGRESSION

The concept that dietary fat plays a major role in the etiology of human breast cancer has evolved over the past 20 years. Extensive studies on different proliferative response of linoleate in cultures have been carried
out with mammary epithelial cells and human breast epithelial cells (Balakrishnan et al., 1989). Fats affect experimental mammary carcinogenesis both by acting as tumour "promoters" and by modulating the effect of fats on the metabolism of carcinogens into reactive intermediates (Karmali, 1983).

4.1.1 Animal Studies

In animal studies, PUFA has been shown to promote development of mammary tumours more effectively than saturated fat. Tumour-enhancing effects of PUFA were reported in transplantable (Rao and Abraham 1976) and spontaneous mammary tumorigenesis (Tinsley et al., 1981). Detailed analysis of the various diets used indicates that the principal PUFA studied was 18:2 n-6.

4.1.2 Mechanism of Action of Dietary fat

It has been postulated that the effect of dietary fat may be mediated by a change in immunocompetence. A high diet in PUFA is known to exert immunosuppressive effects (Vitale and Broitman 1981). Recent evidence in the animal tumours suggests that the tumour-promoting effects of high PUFA diet are mediated through prostaglandins (PG) and in particular, products of arachidonic acid. In DMBA-induced mammary tumorigenesis, when indomethacin, a cyclooxygenase inhibitor, was included in a high PUFA diet, the promotional effects of the high PUFA diet were reduced. Studies reported by number of investigators suggest that inhibition of arachidonic acid metabolism offers a promising new approach to inhibit mammary tumorigenesis (Leaper et al., 1980; Kollmorgen et al., 1983).
To investigate the ability of lung epithelial cells to utilise exogenous 18:2 n-6, a primary monolayer culture system for normal lung epithelial cells was used. This system confers the advantages of a controlled environment, free of complex cellular interactions found *in vivo*.

Studies with lung epithelial cells have indicated that dietary 18:2 n-6 has the capability to promote growth of the cells *in vitro* synergistically with other mitogens. The requirement for 18:2 n-6 to achieve proliferation may be due to the direct effect of 18:2 n-6 on membrane functions, namely maintenance of membrane fluidity and factors arising from the metabolism of 18:2 n-6.

### 4.1.3 Growth Stimulation by 18:2 n-6

[^H]thymidine data showed that 18:2 n-6 synergistically promotes growth specifically when present with I and CT, while in the absence of 18:2 n-6, I and CT were capable of maintaining the cells in the basal condition (Fig. 3.2). In another work with lung epithelial cells from this laboratory, a combination of I, CT, F synergistically promote proliferation was identified (Maya et al., 1991). This may suggest that lung epithelial cells could be promoted to proliferate by two independent pathways namely I, CT, F (lipid-independent pathway), and I, CT, 18:2 n-6 (lipid-dependent pathway). When 18:2 n-6 was present with I and F, there was no significant increase or inhibition of proliferation. This suggests that cortisol might have an inhibitory effect on the action of 18:2 n-6 or cortisol and 18:2 n-6 could not function synergistically. Since experiments with MC5-5 cells have shown that hydrocortisone inhibits the production of PG (Hong and Levine 1976). These results presented here demonstrate that the inhibition by hydrocortisone is
due to block of the release of arachidonic acid from phospholipids and not due to inhibition of prostaglandin synthetase.

Corticosteroids do inhibit PGE$_2$ formation by mouse fibrosarcoma cells and by rheumatid synovia. Studies with perfused guinea pig lungs, suggested that corticosteroids act by reducing the availability of the substrate for the PG synthetase (Hong and Levine 1976). 18:2 n-6 when present alone in the basal condition did not show any proliferative effect (Fig. 3.2). Although 18:2 n-6 by itself does not stimulate growth, its ability to stimulate the growth of lung epithelial cells containing cholera toxin may suggest that dividing cells quickly become deficient in endogenous 18:2 n-6 or that these two mitogens acting independently through its pathway, may demonstrate a concerted action on the cell leading to maximal proliferation. This kind of synergism between growth factors have been seen in many different studies.

A time course analysis in I, CT, 18:2 n-6 suggested that epithelial cells continue to proliferate maximally up to day 6 and day 8, after which a decrease in $[^3\text{H}]$thymidine incorporation was observed (Fig. 3.5a). This may suggest that dietary lipids sustain the growth of cells only for a short duration in cultures. This is specifically true for primary cultures in serum-free medium.

### 4.1.4 Growth Inhibition by Indomethacin

The availability of the enzyme system in lung epithelial cells for metabolising 18:2 n-6 is responsible for growth stimulation in the lipid-dependent pathway. The study with specific inhibitors namely indomethacin (10 µM) suggested an inhibition of growth in the lipid-dependent medium. This effect of inhibition could be reversed by the addition of exogenous PGE$_2$ (Fig. 3.6). Several investigators have reported that less PGE$_2$ is synthesized
as cell density increases and cultures become confluent (Hong and Levine 1976). Changes during cell growth may be related to a decrease in the concentration of a precursor fatty acid or the accumulation of an inhibitor in the culture medium. Prostaglandins enhance growth and DNA synthesis in epidermal cells. Prostaglandins and other eicosanoids may play a role in certain aspects of the pathobiology of malignant diseases for eg. tumour promotion, cellular proliferation and differentiation (Oliw et al., 1983).

Since the lung is normally predominant site for PG metabolism, the possible importance of prostaglandins and PG metabolism in the pathogenesis and therapy of primary cancers originating in lung tissue is of particular interest.

EFA must achieve their biological effects through the synthesis of a variety of metabolites. In fact, the fundamental relationship between EFA and their PG metabolites was proposed as early as 1968 (Cornwell and Morisaki 1985). It is well established that PGE₂ exerts its physiological and pharmacological effects through binding to specific cell-surface receptors and stimulation of adenylate cyclase (Oliw et al., 1983).

PG raise cAMP in a number of cell types including human synovial fibroblasts, the effects of PGE₂ on the action of synovial activator provided part of the evidence that cyclic nucleotides are involved in the regulation of plasminogen activator (Oliw et al., 1983). 8-BrCAMP has been demonstrated previously to act synergistically with synovial activator and a comparison was made with cholera toxin, another agent known to raise cAMP levels by activating adenylate cyclase. These findings indicate that oxygenated metabolites of linoleic acid formed by cyclooxygenases, act as mediators of the growth enhancement.
The compound that are produced by the oxidation of 20-carbon fatty acids such as arachidonic acid and the level of involvement of these eicosanoids, in cancer occurs at all levels from carcinogen metabolism, to tumour promotion, to the rate of proliferation of tumours to the interaction between the turnover and the host to the metastatic potential of tumours.

Oxygenated metabolites of 18:2 n-6 have previously been implicated in the stimulation of cell growth. 18:2 n-6 can promote the growth of tumours (Balakrishnan et al., 1989) and of various cells in culture, and these effects have been blocked by inhibitors of cyclooxygenase and reconstituted by PGE₂.

This suggests that the operative pathway may mainly be the cyclooxygenase pathway responsible for the production of PGE₂, which in turn may activate the cAMP axis or the production of inositol triphosphates. When indomethacin was added to cultures in I, CT, F, no inhibition of growth was observed suggesting that the proliferative signal is operative through an entirely different pathway through their receptors (Fig.3.6).

4.1.5 Labelling Studies with [¹⁴C] 18:2 n-6

The epithelial membrane phospholipids and their fatty acyl moieties play a role in these processes by controlling the physico-chemical properties of the membrane and by providing precursors for bioactive mediators such as eicosanoids upon cell stimulation. In this respect, the type of fatty acid, which contains maximum amount of the dietary lipid may play a role. The role of 18:2 n-6 in maintaining normal cellular responses, synthesis of precursor
products that activate cell proliferation have been studied in detail using in vitro systems (Balakrishnan et al., 1989).

The distribution of radioactivity of $^{14}$C18:2 n-6 among the major lipid groups as a function of time is presented. Fig. 3.9 & 3.10 illustrates the efficient uptake and incorporation of $^{14}$C18:2 n-6 into neutral and phospholipids and the dramatic decline of radioactivity in all lipid classes after 8 days. This decrease in radioactivity associated with the TG and PL fractions during the initial 8 days in l,CT,18:2 n-6 treated cells can largely be accounted for transfer to the subsequent products.

In the non-proliferative condition namely I, an accumulation of 18:2 n-6 in PL fraction was observed, while in the lipid-dependent and lipid-independent condition there was a decline in 18:2 n-6 label both in TG and PL fraction was observed (Fig. 3.9). This suggests that there is an active turnover of 18:2 n-6 between the TG and PL fractions and that the 18:2 n-6 may be further cleaved from PL fraction and utilised for the production of PGE$_2$. Increased 18:2 n-6 content of the tissue may also result in increased production of PGE$_2$ regulating cell proliferation. In cultures of DMBA-induced rat mammary tumours linoleic acid and oleic acid have been found to increase mitotic rate having a similar effect in mammary cell cultures to rats without tumours (Hillyard and Abraham 1979; Carter et al., 1983).

Exogenous fatty acids accumulated as TG in the element variable, or neutral lipid fraction of cell in culture. TG droplets were formed rapidly in cells when exogenous fatty acids were added to media and these droplets disappeared rapidly from cells when exogenous fatty acids were removed from media. When indomethacin was added to the media, an accumulation of 18:2 n-6 label was observed both in TG and PL fractions suggesting that the subsequent pathways may be blocked for further metabolism.
4.1.6 Analysis of Individual Phospholipids

Analysis of individual PL in this study showed that the maximum incorporation of [\textsuperscript{14}C]18:2 n-6 label was accumulated in the PC fraction, observed in the lipid-dependent medium treated cells (Fig. 3.10). A critical 18:2 n-6 level appeared to be maintained in PL. This may be important for maintaining proper signal transduction upon epithelial cell stimulation and it may explain why the observed functional cellular reactivity remains virtually unaltered despite the gross changes in fatty acid patterns.

Studies with culturing of endothelial cells in media supplemented with 18:2 n-6 results in increased levels of 18:2 n-6 and its elongated products in total membrane PL (Vossen et al., 1993). The present data are consistent with these observations and show in addition, that such a phenomenon is not only present in the total PL, but is also apparent within each individual PL class. In our studies 18:2 n-6 label present incorporated into TG and PL fraction at day 2 was maximum in the lipid-dependent growing condition in comparison to the other proliferative lipid-independent condition. Although the mobilisation of 18:2 n-6 among the TG and PL fraction of cell in these two conditions by day 8 indicate a decrease in 18:2 n-6 labelled in both TG and PL fractions. The decline in 18:2 n-6 label in PL was more in comparison to the decline of label in lipid-independent growth condition.

This may not necessarily suggest that PL synthesis is effected, however since it is a transient phenomena and cells are rapidly proliferative in both these conditions, the indication that the membrane synthesis is taking place is obvious. The decline in 18:2 n-6 label could be explained to the mobilisation of labelled 18:2 n-6 for the synthesis of precursor products more
rapidly in the lipid-dependent condition rather than the lipid-independent growth condition. In the non-proliferative medium, the 18:2 n-6 label shows an increase in the PL fractions between the two time points. The reason may be that in totality, since the cells are not proliferating rapidly, the extent of incorporation of the labelled 18:2 n-6 is minimal and further since 18:2 n-6 is not being mobilised as a result the picture is an overall increase in 18:2 n-6 label in PL fraction. Hence, there appears to be a net balance in the overall picture of this effect which depends on the proliferation of the cells and the rapidity of mobilisation of 18:2 n-6 for precursor synthesis especially in conditions that solely depend on 18:2 n-6 for growth. Another explanation for this phenomena which is observed, may be due to fact that the lipid-dependent condition has cold 18:2 n-6 in the medium and there could be a competition of the cold 18:2 n-6 versus labelled 18:2 n-6. Hence the labelled 18:2 n-6 is only used as a tracer to indicate mobilisation. These cells will respond to 18:2 n-6 at 5 μg or higher concentrations and hence the presence of labelled 18:2 n-6 in molar concentration by itself will not produce growth stimulation.

Human endothelial cells that are enriched with 18:2 n-6, unable to convert 18:2 n-6 to 20:4 and as a consequence 18:2 n-6 replaces 20:4 in the element constant or PL precursor pool (Vossen et al., 1993). In contrast 3T3 cells convert 18:2 n-6 to 20:4 and these cells synthesise increased amount of PGE₂ when they are grown in the presence of added 18:2 n-6 (Stubbs and Smith 1984). These studies with growing cell cultures (Denning et al., 1983) emphasise the importance of the size of the precursor pool in regulating PG synthesis.

In this study, when the cells were subjected to the mitogenic stimuli, precursor 18:2 n-6 is released predominantly from the PL fraction by the
activation of phospholipase A₂. Phospholipid may also contribute arachidonic acid for the biosynthesis of the PGE₂. Once released, 18:2 n-6 undergoes oxidative metabolism into a variety of potent biological products.

4.1.7 Labelling Studies with [³H]glycerol

The incorporation of [³H]glycerol into the neutral and phospholipids of lung epithelial cells was also examined by culturing the cells in the presence of [³H]glycerol. A marginal increase of the label was observed in the non-growing cultures in both TG and PL fractions (Fig.3.11). A similar trend was observed when the individual PL were examined (Fig.3.11). However, in the lipid-dependent condition, there was an increase in the glycerol label both in the TG and PL fractions. These are rapidly proliferating cultures hence a rapid incorporation of [³H] glycerol in the PL fraction was observed namely, PI, PS, PE and PC which also showed similar increases (Fig. 3.11). In the rapidly proliferating cultures (I,CT,18:2 n-6), there is an enhanced incorporation of [³H]glycerol in PC fraction when compared to the basal conditions suggesting membrane synthesis.

The fatty acid changes of the present study support many proposed mechanisms for influence on lung epithelial cells such as changes in

i) membrane fluidity
ii) macromolecule mobility
iii) receptor availability
iv) PG and cAMP/cGMP biosynthesis, all processes which may control cell division. Further more, UFA may be metabolised into eicosanoid namely PGE₂ which activate cell proliferation and may act as initiator. In case of cells that are stimulated to proliferate due to the
presence of 18:2 n-6, then its mobilisation to active precursors is important in maintaining the homeostasis and cell proliferation.

Future questions concerning control of proliferation may be directed towards understanding its implications in normal cell regulation

1. Will specific blockage of that synthetic pathway block or retard cell division?
2. Will provision of that lipid restore cell division?
3. Of crucial importance, will loss or removal of control over that synthesis lead inexorably to an increased rate of growth? "Loss of control" is still the only acceptable definition of the cancer state.

In summary, the molecular pathways controlling cell division have been identified. It will be challenging to understand how these events are integrated with growth control mechanisms and how are they linked to abnormal proliferation and to developmental plans of multicellular eukaryotes.

4.2 ras mRNA EXPRESSION IN LUNG EPITHELIAL CELLS

Unveiling the role of ras oncogenes in neoplastic development should have a major impact on the understanding of the pathogenesis of human cancer. ras genes are likely to play a fundamental role in basic cellular functions based on their high degree of conservation throughout eukaryotic evolution. Independently of their phylogenetic origin, they code for proteins that bind guanine nucleotides, have GTPase activity and are associated with the plasma membrane. These properties along with their significant sequence
homology with G-proteins suggest that ras proteins may participate in the transduction of signals across the cellular membrane.

In mammals, they have been implicated in cellular proliferation and terminal differentiation. Therefore, it is likely that ras proteins may function at a critical cross roads of signal transduction pathways. Sequence analysis of these genes and their products have revealed a high degree of conservation, which suggests that they may play a fundamental role in cellular proliferation.

The location of ras proteins in the inner surface of the cell membrane along with their similarity to G-proteins, has raised the possibility that ras proteins may participate in the transduction of mitogenic signals. These observations support the view that ras proteins participate in signal pathways initiated at the cellular surface.

More recently, it has been suggested that ras genes may play a regulatory role in the phosphatidylinositol pathway. Rodent fibroblasts transformed by different ras oncogenes exhibit elevated steady-state levels [2-3 fold] of PIP₂ and their break down products, the second messenger DAG and IP₃. Similar results have been obtained when NIH3T3 cells carrying an inducible N-ras protooncogene were treated with several growth factors such as bombesin and bradykinin but not with EGF or PDG. These observations have led to the proposal that N-ras p21 proteins may be identical to Gp the putative G-protein that mediates the activation of phospholipase C, the enzyme responsible for the breakdown of PIP₂ into DAG and IP₃ (Barbacid,1987).

These results showed the expression of c-H-ras mRNA in cells treated with both the lipid-independent and lipid-dependent media. This suggests that ras oncogene play a fundamental role in cellular proliferation.
Subsequent studies with the addition of indomethacin to the l,CT,18:2 n-6 condition suppressed the expression of ras oncogene, and this suppression of c-H-ras oncogene was reversed by the addition of exogenous PGE₂ (Fig.3.16 & 3.17a). These results correlates with the proliferative data, suggesting a positive role of c-H-ras in cellular proliferation.

Studies on the expression of ras genes in mammalian cells indicate that they are expressed at low levels in most, if not all cell lineages. Increased expression (upto 8 fold) of ras genes has been reported in actively proliferating tissues such as regenerating rat liver. However, increased levels of ras expression do not always correlate with cellular proliferation. Studies aimed at determining the levels of ras proteins in different rat organs have found the highest levels of expression in brain, whereas proliferating tissues only show limited expression. These findings add further support to the concept that ras genes play a role in basic cellular proliferation and in certain specific functions of terminally differentiated cells (Barbacid, 1987).

A further connection between GF and oncogenes was established by the observation that expression of the normal progenitors of oncogenes (proto-oncogenes) is regulated by GF. The effects of the ras and myc oncogenes were examined as representatives of two classes of genes that have distinct and complementary effects on the cellular phenotype (Bar et al., 1991).

In the present study it was observed that the lipid-independent medium (l,CT,F) causes an increase in the ras oncogene in a similar manner as the lipid-dependent condition and the effect of l,CT,18:2 n-6 is not additive to that of l,CT,F. The observation strongly suggests the involvement of ras in transduction of the signals from these growth factors. The essential role of ras
in these pathways has also been suggested in a previous report. Both insulin receptor and the cortisol receptor have tyrosine residues of putative key substrates is thought to be an initial event in signal transduction. A number of proteins phosphorylated by these growth factor receptors in a ligand-dependent manner have been identified, including phospholipase C type γ, raf oncogene products, phosphatidylinositol kinase, and GAP suggesting the existence of multiple pathways. It is not known at present which components are directly linked to p21 and how p21 is activated. Since I,CT,F and I,CT,18:2 n-6 has no additive effects, a common component may mediate the signals from these factors to ras. From this observation, it is proposed that the involvement of ras in normal growth is an important step in the growth regulation of these cells. Other investigators have also described the important role of inositol phospholipid metabolism in transformation by ras and other oncogenes.

The exact role of cellular ras protein in cell division is unknown; it seems to be a common requirement for the action of a variety of growth factors. The ras protein may therefore represent a common element in the molecular sequence initiated by numerous growth factors. Alternatively, cell division may involve a complex interplay of multiple intracellular signals, only one of which involves cellular ras protein. In either case, it is significant that ras protein apparently has a crucial role shortly before the initiation of DNA synthesis.
4.3 mRNA EXPRESSION OF c-myc BY LUNG EPITHELIAL CELLS

The expression level of c-myc in these cells were next examined. In fact, c-myc has been well known to co-operate with ras oncogenes to transform primary cells. As shown in Fig.3.15a & 3.16, the mRNA levels for these genes are essentially the same among the two conditions tested on lung epithelial cells that the expression of c-myc and c-H-ras mRNA was observed at the same time point (i.e.) at 4 hr. Co-operation of ras genes with other cellular or viral oncogenes in the transformation of a variety of primary cells has been well documented (Land et al., 1983). The mechanism(s) underlying this gene co-operation are unknown. It has been reported that in the study of cultured lung cancer cells, that c-myc gene induced alterations in PKC gene expression, especially that of PKC-β, may play a critical role in the phenotypic changes induced by myc-ras complementation (Barr et al., 1991).

Results with the mRNA expression of c-myc also suggest a possible modulation of this gene in response to these mitogens. The maximal expression of c-myc mRNA was at 4 hr (Fig.3.14a). Similar findings have been reported for fibroblasts stimulated by EGF and TSH (Armelin et al., 1984). In the present study, it has been shown that the c-myc mRNA expression was induced by both the proliferative conditions; lipid-dependent and lipid-independent conditions (Fig. 3.15a), a similar observation was found with the mRNA expression of c-H-ras in these cells (Fig.3.16).

The product of the c-myc proto-oncogene is a highly conserved nuclear phosphoprotein whose expression is closely linked to cellular proliferation and with pathways of differentiation. For example, c-myc expression can partially relieve cells from growth factor dependence, block
differentiation, and provide one-step toward malignancy in whole animals and cultured cells. It has been hypothesized that c-myc acts as a direct transcriptional activator, as a stimulation of DNA replication, and as a component of nuclear RNA export, splicing, or processing (Spencer and Groudine 1991).

Several reports support the idea that c-myc expression is linked to cellular growth state, i.e., c-myc RNA levels are low in quiescent cells, but increase dramatically when cells enter the cell cycle after stimulation by a variety of mitogens (Kelly et al., 1983).

Adult mouse kidney, salivary gland, lung, thymus, spleen and mesenteric lymph nodes contain high levels of c-myc steady-state RNA. Further correlations between the abundance of c-myc mRNA and the degree of cell proliferation have been provided by studies of developing human placenta (Pfiefer-Ohlesson et al., 1984), regenerating murine kidney, and regenerating liver (Thompson et al., 1985). Although these tissue specific expression studies appear to provide a general correlation between proliferation capacity and c-myc RNA levels, it is difficult to assess the proportion of proliferating cells in any particular tissue at a particular time and therefore difficult to interpret these studies in a quantitative manner.

Although there is a large and transient induction of c-myc expression within hours after mitogenic stimulation of quiescent cells, the predominant mode of c-myc expression in proliferating cells is continuous synthesis and rapid degradation throughout interphase (Luscher and Eisenman 1990).

In the presence of the appropriate growth factors, both proliferative competency and c-myc are expressed in cells capable of proliferating.
Constitutive c-myc expression can obviate the requirement for certain growth factors depending on the cell type. These observations also raise the possibility that c-myc induction is a common target onto which many diverse mitogenic signals converge. The question that arises then is whether c-myc induction is an essential step in the growth factor signal pathway, or an accessory event coupled to the mitogenic response. Recently, it has been found that many of the agents that induce c-myc expression share a common interaction with phosphatidylinositol metabolism and PKC, i.e., agents that induce kinase C also induce c-myc expression (Cole, 1986).

It has been postulated that in BalB/3T3 fibroblasts, at least two growth factor signal transduction pathways can induce c-myc mRNA (Wright et al., 1989). One pathway is dependent on PKC; a second pathway is independent on PKC, but dependent on cAMP. The results of stimulation of c-myc mRNA expression by two different growth proliferative conditions agrees with this data, one is I, CT,F condition which is PKC dependent and the other one I, CT, 18:2 n-6 is cAMP dependent or through IP3.

Phosphatidylinositol breakdown products function as cytoplasmic second messengers to induce transcription of c-myc although they may not play an exclusive role in this regard. It has already been shown that both PDGF and lymphocyte mitogens promote a rapid increase in PIP2 turnover within their respective target cells. Other agents that promote PIP2 turnover or that mimic the biochemical effects of PIP2 hydrolysis products (bombesin, calcium ionophores, phorbol-based tumour promoters) will induce c-myc expression in fibroblasts, lymphocytes and other cell types. The PIP2 hydrolysis products, DAG and IP3 function directly and indirectly to activate PKC (Cole, 1986).
4.4 TNF-α mRNA EXPRESSION STUDIES

TNF-α is a cytokine produced by macrophages and a number of other cell types. TNF-α has a broad and diverse biological response, ranging from the induction of necrosis of some transplantable tumours and regulating numerous immunological responses to stimulating cell growth and differentiation.

In lung, a close association between the epithelial cells and the alveolar macrophages is observed and whether such an association between the different cell types may have any effect on stimulation of mitogenesis or immunomodulation needs to be studied in detail. The present report describes that the studies on the in vitro effects of different growth factors and hormones on lung epithelial cells in the production of TNF-α. This data provide evidence that I, CT, F can induce or augment TNF-α production by lung epithelial cells. Cytokines are relatively low molecular weight proteins, many of which were originally discovered to be produced by immune or inflammatory cells although several mesenchymal and epithelial cells are now implicated as potential sources of cytokines (Ulich et al., 1989).

TNF-α mRNA was detected by northern blotting in whole-organ homogenates of the spleen, liver, kidney, lung and small bowel in native and saline-injected control rats, supporting the hypothesis that TNF-mRNA is present in vitro in a preformed intracellular pool (Ulich, et al., 1989). The results demonstrate that lung epithelial cells express TNF-α as detected by dot and northern blot analysis (Fig.3.14a).

The amplification of TNF-α production by these growth factors can be crucial importance in the process of lung inflammation and fibrogenic
disease such as found in silicosis and asbestosis (Dubois et al., 1989). These observations provide new insight into the mechanism by which these growth factors may modulate the production of inflammatory and fibrogenic cytokines.

It has also been shown that TNF-α is not produced by unstimulated cells as well as by the cells grown in lipid-dependent condition. This shows that 18:2 n-6 can modulate TNF-α gene expression and suppress the production of TNF-α. Although many potential mechanisms exist for intracellular signal resulting in PGE₂ induced suppression, a prime candidate in this situation is cAMP. Previous studies have demonstrated that PG of the E series are effective stimuli for the rapid induction of intracellular cAMP in a dose-dependent manner. It has already been shown that factors like PGE₂, prostacyclin are known to down-regulate TNF-α mRNA expression. Northern blot and in situ hybridization analysis showed that PGE₂ serves as a regulatory mediator and that TNF production can be modulated at the level of transcription (Wong and Goedal 1989). This evidence suggests that PGE₂ may serve as a potent regulator of TNF-α production. These investigations have significance light of the multifunctional role of TNF-α in various facets of immune pathophysiologic responses.

4.5 INOSITOL PHOSPHATE MOBILISATION

Cell growth can be triggered by a large number of mitogenic signals that include fertilization, conventional neurotransmitters and hormones, and a large number of growth factors. The fact that phorbol esters display remarkable synergistic interactions with many growth factors (Berridge, 1984)
suggests that the C-kinase pathway and by inference the inositol lipids, may play a fundamental role in regulating cellular proliferation.

The two major ionic events that contribute to this onset of proliferation are changes in the level of calcium and the activation of a neutral Na⁺/H⁺ exchange carrier. Multifunctional calcium mobilising receptors may be responsible for initiating both the ionic events as well as the increase in cAMP that contribute to the onset of proliferation. Changes in the phosphoinositide metabolism have been described upon activation of cultured cells (Berridge, 1984) and lymphocytes by a variety of mitogens. Much of this literature on the possible relationship between phosphoinositide metabolism and cell growth has been studied in detail.

In common with many other cell types where Ca²⁺ apparently acts as an intracellular messenger, the Ca²⁺ mobilised by hormones is initially derived from intracellular stores, probably the endoplasmic reticulum and mitochondria, followed by a supplementary influx of Ca²⁺ across the plasma membrane which serves to maintain the elevation of cytosolic free Ca²⁺. The mechanism by which hormones mobilise intracellular Ca²⁺ is currently unknown, but since there is no requirement for hormones to cross the plasma membrane, it is apparent that same form of the transducing mechanisms or "second messenger" must exist to relay the hormonal signal to its intracellular sites of action.

It was noted that hormone induced Ca²⁺ mobilisation is associated with alterations of inositol lipid metabolism in a wide variety of tissues. More recently, it has been shown that Ins IP₂ and IP₃ are released in parallel with the breakdown of polyphosphoinositides in insect salivary glands, rat brain, platelet cells, and platelets. Changes in the levels of almost every intermediate of hormone-stimulated inositol lipid metabolism have been suggested to
play a role in Ca\textsuperscript{2+} mobilisation, including cyclic IP, PtdIns, phosphatidic acid, polyphosphoinositides, DAG, arachidonic acid and inositol phosphates (Thomas et al., 1984).

The purpose of the present study was to extend previous investigations into the possible involvement of inositol lipid metabolism in hormone induced Ca\textsuperscript{2+} mobilisation in lung epithelial cells.

The data presented here describe the changes in inositol polyphosphate levels which accompany the growth factor stimulated inositol lipid breakdown in lung epithelial cells concomitant with the breakdown of PIP\textsubscript{2} which is induced by the lipid-dependent condition treatment of cells, there is a rapid accumulation of IP\textsubscript{3}. Since the only known mechanism for IP\textsubscript{3} formation in mammalian cells is through a phosphodiesterase cleavage of PIP\textsubscript{2}, these data indicate that the major route for hormone induced PIP\textsubscript{2} breakdown is through this phosphodiesterase (Phospholipase C).

IP\textsubscript{4} level increases at a similar rate to the levels of IP\textsubscript{3}. These data are compatible with either a direct breakdown of PIP\textsubscript{2} to IP\textsubscript{4} or with a situation where PIP decreases as a result of increased PIP\textsubscript{2} resynthesis while IP\textsubscript{4} is formed by degradation of IP\textsubscript{n}. The latter possibility appears to be more likely because it is clear from this data that the break down of PIP\textsubscript{2} is partially compensated by an increased rate of re-synthesis of this PL from PIP and PI. This conclusion is based on the observation that IP\textsubscript{3} continues to accumulate for several minutes after PIP\textsubscript{2} levels have declined to a new steady state.

The concept that polyphosphoinositides are maintained at a new steady state, with the rate of hormone stimulated break down matched by an increased rate of resynthesis, is further supported by the finding that Ptd Ins breakdown continues for several minutes at essentially the same rate as that
observed for the first few seconds of polyphosphoinositide breakdown (Thomas et al., 1984) while no detectable increase of IP occurs during these early times after growth factor addition.

Hydrolysis of PtdIns by its phosphodiesterase yields a mixture of inositol 1:2 cyclic phosphate and IP; P1P gives IP2 and PIP2 will produce IP3. The existence of such inositol phosphates was first discovered in 1968, showed that a crude mitochondrial fraction of brain contained IP, IP2 and traces of IP3.

Acetylcholine stimulated a small increase in the levels of IP and IP2. An increase in these two inositol phosphates has also been described in guinea-pig synaptosomes after treatment with ionophore A23187. A slightly different pattern was described in rabbit cris smooth muscle, where acetylcholine stimulated an increase in the release of IP and IP3 with no change in the level of IP2 (Berridge et al., 1983).

There was a 10 fold increase in the level of IP2 after stimulation with I,CT,F for 10 min. I,CT,F induced an increase in the levels of IP2 and IPn in the lung epithelial cells (Fig.3.18 & 19). Such large increases in the levels of these two IP's would seem to suggest that one of the primary biochemical actions of the growth factors is to stimulate the hydrolysis of the polyphosphoinositides by a phosphodiesterase.

An increase in the appearance of IP3 thus consistent with the idea that the mitogens are acting to stimulate the hydrolysis of a polyphosphoinositide through a phosphodiesterase rather than through a phosphomonoesterase pathway. However, the picture is complicated by the fact that these mitogens also increased the level of IP and free [3H]inositol in
the case of \( \text{I,CT,F} \) stimulation, which is presumably produced from IP by phosphomonoesterase action (Fig. 3.18).

This IP could be formed directly by the action of phosphomonoesterase on the IP\(_2\) and IP\(_3\). Although the failure to detect inositol 1:2 cyclic phosphate could suggest that the latter pathway operates, it is known that many tissues contain an active inositol 1:2 cyclic phosphate-2-phosphohydrolase, which could rapidly convert the cyclic derivative into IP.

According to the recent investigation on the relationship between proto-oncogene and the inositol lipids, the \text{src} and \text{ros} gene products may play a role as kinase for inositol PL, PtdIns, P1P (Kubota et al., 1986).

The enzymatic reactions associated with this bifurcating signal pathway based on the inositol lipids may represent the sites of action of certain oncogenes. The \text{sis} oncogene is responsible for producing PDGF, known to stimulate inositol lipid metabolism. The \text{erb-B} gene codes for a truncated EGF receptor. Proteins encoded by the \text{src} and \text{ros} oncogenes may function as inositol lipid kinases to convert PI into the PIP\(_2\) used by the receptor mechanism. It is reasonable to speculate that other oncogenes, whose functions are still unknown, may code for enzymes concerned with the remaining steps of this bifurcating signal pathway (Berridge et al., 1984).

The discovery of IP\(_3\) as an intracellular second messenger may contribute to our understanding of those aspects of cell growth and development that depend on the mobilization of calcium from intracellular stores. It has been shown that \( \gamma \text{IGF-II} \) stimulates calcium influx in BalB/C 3T3 cells primed with EGF. It has also been shown that IGF-II stimulated
production of IP$_3$ in proximal tubular basolateral membranes from canine kidney (Rogers and Hammerman 1988).

IP$_3$ is also known to be a DNA synthesis stimulator. For example, hyperstimulation of the parotid via the cholinergic parasympathetic pathway, which results in a large accumulation of IP$_3$ also stimulates DNA synthesis (Berridge et al., 1984).

It has been proposed that IP$_4$ may function to regulate Ca$^{2+}$ entry across the plasma membrane (Joseph et al., 1987). More recently, it has been shown that IP$_2$ and IP$_3$ are released in parallel with the breakdown of polyphosphoinositides in insect salivary glands, rat brain parotid cells and platelets (Thomas et al., 1984). The present study supports the concept that in lung epithelial cells the regulation of phosphatidylinositol synthesis is tightly regulated and is unlikely to be affected by even considerable changes in cellular inositol content.

In addition to their role in the rapid responses of cells to hormonal stimulation, the products of phosphoinositide hydrolysis also participate in the regulation of intracellular events involved in cell growth and proliferation. In addition to stimulating phosphoinositide hydrolysis, several growth factors and oncogenic products with tyrosine kinase activity are associated with the PI kinase activity that promotes the synthesis of plasma membrane phosphoinositides. The mechanism of action of insulin also involves several facets of phospholipid production and metabolism (Catt and Balla 1989).

In LCT, 18:2 n-6 treated lung epithelial cells, the levels of IP$_3$ and IP$_4$ increased rapidly at 20 min. It has been shown that IP$_4$ is formed as rapidly as IP$_3$ in various stimulated tissues and cells (Batty et al., 1985) and Irvine and Berridge (1984) presented evidence for a novel ATP dependent kinase that
converts IP₃ to IP₄. Because the absolute rates of synthesis and degradation of IP₃ and of IP₄ in stimulated cells appear to be of the same order at least in the parotid gland (Irvine et al., 1986), the pathway for metabolism of IP₃ through IP₄ to IP₃ and hence for its inactivation as a Ca²⁺, mobilising ligand, may be quantitatively as important as its dephosphorylation to IP₂.

The data in Fig. 3.21-23 suggested an association between the 1,CT, 18:2 n-6 stimulated release and metabolism of arachidonic acid and the early stimulation of PI synthesis. In order to determine if a relationship indeed existed, the effect of indomethacin was examined on these activities (Fig. 3.21, 22 & 23). Fig. 3.21-23 demonstrated that inhibition of this metabolism occurred at approximately 10 μM indomethacin consistent with the previous reports. It has already been shown that indomethacin inhibits thrombin-stimulated PI synthesis in cultured fibroblasts (Hallman, 1984). It has already been demonstrated that PGE₂ can cause phosphoinositide breakdown with the resultant formation of IP₃, IP₂ and IP in a time and dose-dependent manner (Yokohama et al., 1988).

It seems likely therefore, that the stimulation of PI synthesis is a secondary event resulting from the cyclooxygenase mediated metabolism of arachidonic acid. This is consistent with the evidence implicated cyclooxygenase mediated products of arachidonic metabolism in the thrombin stimulation of phosphatidic acid in platelets (Yokohama et al., 1988).

The present study confirms that, in common with other cells, stimulation by 1,CT,18:2 n-6 in lung epithelial cells leads to a marked hydrolysis of phosphoinositides and accumulation of inositol phosphates. An earlier study of BTSM reported similar findings. Almost certainly the major substrate, at least over the first few minutes of growth factor stimulation is
PIP₂, with the production of IP₃, which then undergoes dephosphorylation to IP or phosphorylation to IP₄ and subsequent dephosphorylation to appropriate bis and mono-phosphates (Chilvers et al., 1989). This would be consistent with a role for IP₃ and perhaps IP₄ in lung epithelial cell Ca²⁺ homoeostasis.

Although stimulated labelling of the inositol lipids would be expected as a consequence of growth factor-induced hydrolysis and indeed formed the basis of the first observation of a "phosphoinositide response" by Hokin and Hokin (1953) and many other early studies (Michell 1975), the extent of growth factor induced [³H]inositol labelling in lung epithelial cells is considerable.

In general, inclusion of cholera toxin in tissue culture results in enhanced proliferation of lung epithelial cells. Like other bacterial toxins (Murphy and Rozengurt, 1992), CT may selectively potentiate the action of G-protein linked receptors and effectively stimulated the phosphoinositide system and a rise in intracellular calcium.

The results also indicate that linoleic acid treated cells showed increased levels of IP₃, while such as increase in IP₃ levels were not associated in cells treated with cortisone. This imply that the proliferation inducible signals in cortisone treated cells may be either IP₃-independent or changes in IP₃ levels could be very rapid and transient.

Indomethacin, a powerful inhibitor of arachidonic acid metabolism, inhibited the inositol levels, the expression of c-myc, c-H-ras and the overall proliferation of lung epithelial cells. This is interesting in the context that the metabolites of arachidonic acid were known to activate many intracellular messengers such as cAMP and PKC and increase Ca²⁺ levels (McPhail et al.,
1984). As such the inhibitory effect of indomethacin on cell proliferation may be related to multifaceted alterations in cell signalling processes (Fig. 4.1).

Proliferation of lung epithelial cells is well correlated with the increases in hydrolysed inositol levels and expression of immediate early genes such as c-myc, c-H-ras etc. Many growth factors utilise the messenger IP₃ to set up prolonged calcium signals, often organised in an oscillatory pattern. An elevation of intracellular IP₃ exerts a profound effect on calcium signalling by controlling both the release of stored calcium (from the internal organelles e.g., endoplasmic reticulum) and the influx of extracellular calcium across the plasma membrane. One function of this calcium signal is to activate the immediate early genes responsible for inducing resting cells (G₀) to re-enter the cell cycle. Calcium is also implicated in delivering appropriate signals for the initiation of DNA synthesis at the G₁/S transition and completion of the cell cycle by stimulating events at mitosis. The present study thus summarises the effect of growth factors in cell proliferation where phosphoinositide metabolism plays a significant role.

CONCLUSIONS

An in vitro model for understanding the intricacies involved in the growth regulation of normal lung epithelial cells has been developed. Using which the utilisation of 18:2 n-6 and other mitogens were evaluated.

The following are the important highlights of this study:

- Two growth conditions have been identified which induces maximum proliferation of lung epithelial cells in culture.
  a. Lipid-independent condition (l, CT, F)
  b. Lipid-dependent condition (l, CT, 18:2 n-6)
Fig. 4.1 Model to illustrate the proposed role of phosphoinositides in the action of mitogenic signals.
• The possible mode of stimulation by I,CT,F and I,CT,18:2 n-6 are different, probably operating through entirely different pathways.

• Time course analysis showed a maximum growth of lung epithelial cells in the I,CT,18:2 n-6 condition between day 6 and day 8.

• I,CT,18:2 n-6 induced growth of lung epithelial cells was inhibited in the presence of indomethacin and when exogenous PGE2 was added, a reversal of proliferation is observed.

• Pre-labelled studies with $[^{14}C]18:2$ n-6 have shown distinct turnover of 18:2 n-6 between TG and PL fractions in the cells with the two proliferative media. Maximum incorporation was found in PC fraction.

• $[2^{-3}H]$glycerol labelling studies showed that in the I,CT,18:2 n-6 treated cells, there was an accumulation of the label in TG and PL fractions between the two time points.

• Lipid-independent and lipid-dependent media induces the expression of c-myc and c-H-ras by 4 hr time point.

• TNF-α mRNA expression was induced by 10 hr in the lipid-independent medium but not by the lipid-dependent medium.

• In the basal conditions, the levels of inositol phosphates did not increase significantly except for a marginal increase in the levels of free inositol, IP and IP$_4$.

• In the lipid-dependent condition, the levels of IP$_2$ and IP$_n$ increased rapidly within 10 min and free inositol by 20 min.

• The levels of IP$_n$ increased in the lipid-dependent condition at 10 min and the levels of IP$_3$ and IP$_4$ increased rapidly thereafter by 20 min. In the presence of indomethacin there was an inhibition in the accumulation of all the inositol phosphates.