Isolation and Characterization of
A Growth Regulatory Polypeptide
from Cultured 3T3 Fibroblasts

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ABSTRACT

The growth rate of most normal cells in culture decreases as the number of cells reaches a characteristic saturation density. This phenomenon, termed density-dependent inhibition of growth, was first described and studied extensively in the cultures of 3T3 fibroblasts. Previous studies have demonstrated that medium conditioned by exposure to cultures of density-inhibited 3T3 fibroblast contained a growth-inhibitory activity that acted on sparse proliferating cultures of the same cells.

This thesis describes the purification, to apparent homogeneity, of the growth inhibitory activity by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. The highly purified preparation yielded a single polypeptide (M_r 13,000) in sodium dodecyl sulfate polyacrylamide gel electrophoresis under both reducing and non-reducing conditions. This component in designated FGR-s (13K), which stands for Fibroblast Growth Regulator - soluble form (M_r 13,000).

The inhibition of cell proliferation by FGR-s (13K) can be monitored by two independent assays: (a) quantitation of S phase DNA synthesis as determined by the incorporation of radioactive thymidine; and (b) assessment of cell number. The dose-response curve of growth inhibition by FGR-s (13K) on 3T3 target cells showed that 50% inhibition was achieved at a concentration of 3 ng/ml (2.3×10^{-10} M). The inhibition was reversible and cannot be ascribed to any cytotoxic

effects.

To ascertain that the growth inhibitory activity of FGR-s (13K) is due to the M_r 13,000 polypeptide observed in the highly purified preparation, hybridoma clones secreting monoclonal antibodies directed against FGR-s were generated by *in vitro* immunization of rat splenocytes. One monoclonal antibody, designated Antibody 2A4, specifically bound FGR-s (13K). The growth inhibitory activity of FGR-s (13K) was depleted upon passing the purified preparation over an affinity column containing Antibody 2A4. In addition, Antibody 2A4 neutralized the activity of FGR-s in growth inhibition assays. These results indicate that the M_r 13,000 polypeptide is responsible for growth inhibition.

The addition of Antibody 2A4 to 3T3 cell cultures in the absence of any exogenously added FGR-s also enhanced the level of DNA synthesis in a dose-dependent fashion. This suggests the possibility that Antibody 2A4 may be neutralizaing the activity of FGR-s (13K) molecules endogenous to the culture and may be partially reversing the effects of density inhibition. The results also imply that the FGR-s (13K) molecule may play a role in the normal mechanisms of density dependent inhibition of growth.

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ABBREVIATION

BSA	Bovine Serum Albumin
DME	Dulbecco Modified Eagle's Medium
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FGR-s	Fibroblast Growth Regulator--soluble form
FGR-s (13 K)	Component of FGR-s, M_r 13,000
G_1	Gap one
G_2	Gap two
IGFs	Insulin-like Growth Factors
M	Mitosis
MSA	Multiplication Stimulating Activity
NGF	Nerve Growth Factor
NS-1	Mouse Myeloma Cell Line NS-1 Ag4/1
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived Growth Factor
S	DNA Synthesis
TCA	Trichloroacetic Acid
SDS	Sodium Dodecyl Sulfate
TGFs	Transforming Growth Factors

INTRODUCTION

A cell's life is usually described in terms of the cell cycle. This cycle is divided into four major parts (Figure 1): a G_1 phase, an S phase, a G_2 phase, and an M phase. Both biochemically and morphologically, the two parts most extensively characterized are the S phase which is defined as the period for DNA synthesis and chromosomal replication, and the M phase which is the time for cell division.

After mitosis, each daughter cell goes through a G_1 phase in preparation for DNA synthesis and another round of cell cycle. Somewhere between the end of mitosis and the onset of next S phase, there occurs a point at which a cell has to decide, depending upon a host of enviromental factors, whether to go on for another round of the cycle. If the decision is not to go for another round, then it will stop at the decision point, which is sometimes called the G_0 phase. If the decision is go for another round, then the cell will make all the preparations necessary for DNA synthesis and mitosis.

Most normal cell have these two alternative modes of existence. One is the proliferative mode which is represented by the large circle; the other is the quiescent mode, which is represented by the small circle (Figure 1).

A number of in vitro systems have been developed to study the transition of cultured cells from the quiescent state to the proliferative state. Examples of these systems include the stimulatory actions of epidermal growth factor, fibroblast growth factor,

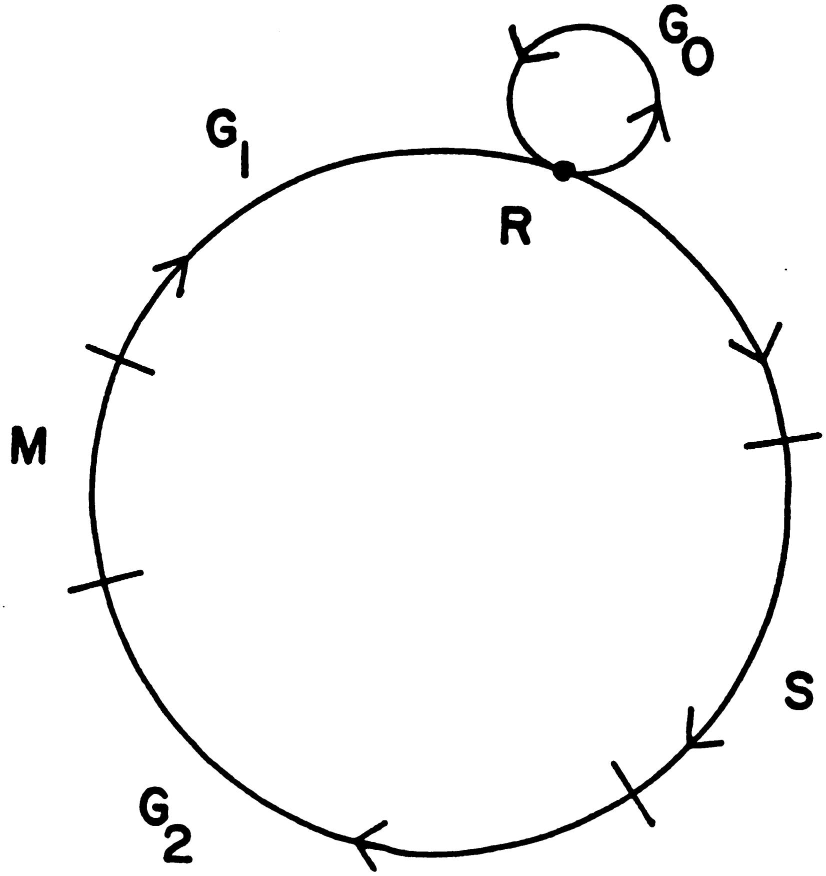

Figure 1. Schematic representation of cell cycle.

platelet-derived growth factor, and other polypeptides which are in general known as mitogens. In contrast, much less progress has been made to study the transition from proliferative state to quiescennt state. One example of this transition is the well known phenomenon called density-dependent inhibition of cell growth. This phenomenon was best demonstrated in 3T3 cultures, a cell line originally derived from mouse embryonic fibroblasts. Cultured 3T3 cells possess the property of being highly sensitive to density-dependent inhibition of growth.

Previously, this laboratory has been able to demonstrate that medium conditioned by exposure to cultures of density-inhibited 3T3 fibroblasts contained a growth-inhibitory activity that acted on sparse proliferating cultures of the same cells. This activity was fractionated, resulting in a preparation called Fibroblast Growth Regulator - soluble form (FGR-s) that contained two major polypeptides (M_rs 10,000 and 13,000).

This thesis work continues the previous efforts to identify and to purify the inhibitory activity in the FGR-s preparation. The successful attainment of this growth inhibitory factor in homogeneous form will allow the studies of negative growth regulation to be carried out at the molecular level. This, in turn, will help to understand the coordinated interactions between growth stimulatory factors and growth inhibitory factors in the total network of growth control.

CHARPTER 1

LITERATURE REVIEW

Growth is essential for development and maintenance of normal multicellular organisms. The mechanisms that regulate growth are not only selective for cells in different types of tissue but also responsive to the fluctuations in the enviroment. Similar forms of growth regulation, to a certain extent, have been observed in cell cultures. Generally, normal cells show more restricted and ordered growth than their transformed counterpart. The extent to which the growth regulation is lost in cell cultures correlates with the tumorigenecity of these cells *in vivo* (1). This observation suggests that the growth regulation observed *in vitro* may have relevance *in vivo*.

It is clear that many different factors can control growth. In cell cultures, besides the topographic considerations (2,3), these controlling factors can be categorized into two groups. One is macromolecular factors derived from serum and/or elaborated from the cultured cell itself (4,5). Another is low molecular weight nutrients and metabolites derived from the medium or cultured cell (6). It has been proposed that cells are arrested at a point in the G_1 phase of the cell cycle, known as the "restriction point", whether the controlling factor(s) belongs to low molecular weight component or the macromolecular category (7). Growth resumes when these factor(s) are added to the culture medium (8,9).

Macromolecular factors that are responsible for growth regulation in cell cultures can be categorized into two groups: (a) growth stimulatory factors; (b) growth inhibitory factors. The control of cell proliferation resides in the balance between these two opposing forces of mitogenic and inhibitory factors. Therefore, to study the growth regulation by endogenous inhibitory factors, one must be constantly aware of the activities of growth stimulatory factors.

Over the past decade major progress has been made in the studies of the growth stimulatory factors (10-13). These include the identification and isolation of a large number of growth stimulatory factors, structural analysis of some of these factors in terms of amino acid, mRNA, and genomic sequences, and receptor characterization for some of the growth factors in terms of their structure and function. This research has made it possible to converge studies on growth regulation and those on oncogene products (14-17).

Much less progress has been made in the studies of growth inhibitory factors when compared to the status of growth stimulatory factors. This is mainly due to a lack of a well-defined experimental paradigm to study their molecular nature as well as the technical difficulties inherently associated with many of the assay procedures used to detect inhibitors of cell proliferation.

This review will briefly describe (i) some of the well characterized growth factors with the attempt to categorize these factors into a limited number of "families" (Table 1); (ii) mechanisms of growth factor stimulation and their relationships to oncogenesis; (iii) recent progress in isolation and characterization of growth inhibitory factors.

Table I. Growth factors and their families.

Family	Member	Source	Mol. Wt.	Mol. St.	References
EGF	EGF	human urine, mouse submaxillary gland	6,054	monomer	18-21
	TGF-α	MuSV transformed cells, platelets	7,000	monomer	56-59
	Related	retrovirus transformed cells			66
PDGF	PDGF	platelets, serum, placenta, etc.	28,000 31,000	dimer	67
	$P24^{sis}$	SSV transformed cells	24,000	dimer	81-83
	ODGF	CM of osteosarcoma	31,000	dimer	75,76
	Glioma-DGF	CM of glioma cell culture	31,000	dimer	77
	Related	CM of SV40 transformed cell cultures			78
Insulin	Insulin	β-cell of pancreas	5,700	dimer	86,87
	IGF-I	plasma	6,000	dimer	94
	IGF-II	plasma	6,000	dimer	98
	MSA	CM of liver cell culture	6,000	dimer	90
	NGF	mouse submaxillary gland, snake venom	13,000	dimer	106,107
	Relaxin	glanulosa cells of corporsa leuta	6,000	dimer	114

Abbreviations used are: Mol. Wt., molecular weight; Mol. St., molecular structure; EGF, epidermal growth factor; TGF, transforming growth factor; MuSV, murine sarcoma virus; PDGF, platelet-derived growth factor; SSV, simian sarcoma virus; ODGF, osteosarcoma-derived growth factor; CM, conditioned medium; Glioma-DGF, glioma-derived growth factor; SV40, simian virus 40; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; MSA, multiplication stimulating activity; NGF, nerve growth factor.

Growth Factors and their Families.

Epidermal growth factor (EGF). EGF represents one of the best characterized growth factors in terms of the availability of highly purified preparation (18-21), primary structure (22-24), membrane receptor (25-30), target cell spectrum (31-35), fate after receptor binding (36-39), and its related polypeptides (40,41). EGF consists of a single chain polypeptide, with 53 amino acid residues (M_r 6,045). Effective mitogenic concentration is at ng/ml level (18,19,42). Its mitogenic effect is not restricted to epidermal cells. Fibroblasts, both established cell lines such as the mouse 3T3 cell and human diploid fibroblasts in their early passage, respond to EGF (43-46). The cell surface receptor for EGF has been identified and characterized (M_r 170,000) (47-49). Antibodies to EGF receptor have been shown to mimic the mitogenic activity of EGF (50). The EGF receptor contains, as an integral part of its structure, a tyrosine-specific protein kinase activity capable of phosphorylating itself and a variety of other proximal cellular substrates (51,52). Nucleic acid sequence studies have shown homology between the v-erb oncogene and the EGF receptor. The oncogene product is a truncated EGF receptor, lacking the ligand-binding domain exposed to the outside of the cell. This relationship suggests a connection between growth factors and transformation processes in some virally infected cell cultures (17).

TGF-α, found as one of the two components of sarcoma growth factors (53,54) (another component is TGF-ß), shows about 40% amino acid sequence homology with mouse EGF (17.55), implying that these two growth factors may have a common ancestral origin but have diverged substantially since.

Like EGF, production of TGF-α is not restricted to MuSV-transformed cells; rather, it is a property of several transformed cell lines (56-59) as well as normal platelets (60,61). There are at least three other lines of evidence that suggest the relatedness of EGF and TGF-α: (a) receptor cross-reactivity: TGF-α binds to EGF receptor without significant affinity difference (62,63,65); (b) both TGF-α and EGF potentiate TGF-β for colony formation in soft agar cultures (43,60); and (c) both are derived from similar larger precursor molecules: the EGF precursor is about 20 times as large as EGF (64,65). TGF-α appears to share a similar precursor. Finally, by using antibodies against a synthetic fragment of TGF-α, a number of TGF- related polypeptides have been found (66).

Platelet-derived growth factor (PDGF). PDGF, the principal mitogen in serum for cultured cells of mesenchymal origin, is a heat-stable, cationic (pI ~ 9.8) protein (M_r 28,000-30,000) (67). Upon reduction, PDGF yields two chains (A chain, M_r 15,000; B chain, M_r 14,000) suggesting that the intact molecule may consist of two polypeptide chains held together by disulfide bonds (67,68). PDGF, originally isolated from whole human serum (69) turned out to be a component released from the α-granule of platelet upon clotting (68,70-73). PDGF is a potent mitogen exerting its effects at ng/ml concentrations (70). It stimulates DNA synthesis and mitosis in cultures of Balb/c-3T3 fibroblast, human diploid cells, aortic smooth muscle cells, glial cell (70,71). While a great number of cell types respond to PDGF, it is suggested that its major function in vivo may be repairing damage to the vascular lining--mainly due to the observation that phylogentically appearance of PDGF-like activity in clotted blood serum coincides with the appearance of the pressurized vascular system in vertebrates (74).

Unlike normal cells, cultures of viral transformed fibroblasts and human osteosarcoma cells do not require PDGF for growth. Based on this idea, a series of PDGF-liked polypeptide growth factors were identified in a variety of transformed cell lines. These include osteosarcoma-derived growth factor (ODGF) (75,76), fibroblast-derived growth factor (FDGF) (77,78), glioma-derived growth factor (76,79), and a PDGF-like growth factor from SV40 transformed baby hamster kidney (BHK) cells (78,80). FDGF was originally identified in an invasive tumor cell line (SV28) as a fibroblast migration factor (77) and was able to stimulate the proliferation of normal 3T3 cells in low serum concentrations at which only transformed 3T3 cells proliferate, and to stimulate the growth of 3T3 cultures to higher saturation density (77). Both ODGF and FDGF have similar physico-chemical properties as PDGF. Both have been identified as M_r 31,000 components composed of two polypeptide chains of similar apparent molecular weights (75,78). Also, they cross-react with PDGF in binding to PDGF receptor as well as antibodies to PDGF (75,78). Another series of transforming polypeptides related to PDGF have been found in the cytoplasm of simian sarcoma virus (SSV) transformed cells, namely $p28^{sis}$ and $p24^{sis}$ (76,81-83).

Insulin. Insulin has attracted the most attention among the major growth factors--not because of its growth stimulatory activity (84) but its spetacular action on carbohydrate metabolism (85). Insulin, (M_r 5,700), is well known for its heterodimeric structure which consists of A and B chains held together by three disulfide bonds. This is derived from a larger precursor proinsulin (M_r 6,000) by proteolysis (86,87).

While it has been demonstrated that insulin has mitogenic effect on a number of cell types, very high concentrations are required, and the total mitogenic effect is, in general, much smaller compared with the effect of serum. For example in human foreskin fibroblasts, 20 ug/ml of insulin are required to initiate DNA synthesis in subconfluent cultures to an extent of 1/20 of that observed with serum (84). The idea that insulin could indeed be a growth factor was supported by the observation that most of the mitogenic peptides isolated from blood or serum have an insulin-like activity (88-91). However, the expectation that insulin is a physiologically significant mitogenic agent has yet to be verified. The fact that insulin potentiates the stimulatory effect of other growth factors, although it has little effect per se (92,93), supported the idea that physiologically insulin actually works in concert with other growth factors.

Insulin related growth factors include: nerve growth factor (NGF), relaxin, and insulin-like growth factors (IGFs) (94-98). IGFs are a family of polypeptides that are structural homologous to and functionally mimic insulin, but are immunologically distinct from insulin (99). These include IGF-I, IGF-II, somatomedins, and multiplication stimulating activity (MSA).

IGF-I and IGF-II are polypeptides of 70 and 67 amino acid residues, respectively. The polypeptide structures of IGF-I and IGF-II are similar to that of insulin which consists of A and B chains held together by three disulfide linkages. Genomic sequence analysis suggests that both IGFs are derived from preproinsulin-like precursors. IGF-I and IGF-II share 62% homology to each other and 50% homology to insulin in primary amino acid sequence. Based on limited structural and immunological data, the IGFs appear to be identical to somatomedins (99,100), the presumed mediators of the effect of growth hormones, and the growth factors secreted by liver cells in culture including multiplicating stimulation activity (MSA) (90).

Insulin, IGF-I, and IGF-II crossreact to all three of their receptors, but with differing affinity (101-103). Insulin has highest affinity to its own receptor, lower affinity for IGF-I receptor and almost no affinity to IGF-II receptor. For IGF-II, the converse is true. IGF-I has a moderate affinity for all three receptors.

In spite of the structural similarity, IGFs and insulin apparently are responsible for dinstinct physiological functions. The metabolic responses (such as glucose oxidation) of all three polypeptide correlated well with their affinity to the insulin receptor, suggesting that insulin receptors (bound differentially by all three polypeptides) are responsible for these responses (104). The growth-stimulatory effects of these polypeptides correlated better with their affinity for the IGF-I and IGF-II receptors, leading to the hypothesis that long-term effects of insulin may be mediated via IGF receptors (105).

Nerve growth factor (NGF) was first identified as a constituent of mouse sarcomas in experiments designed to test the effect of these tumors on the development of the sympathetic nervous system (106,107). It was subsequently shown to be present in much higher concentrations in snake venom (108) and in the submaxillary gland of adult male mice (108). Purified NGF consists of two polypeptide chains, with identical sequences of 118 amino acid residues, held together by three disulfide bonds (109). NGF and insulin share common regions of sequence, which may indicate a common ancestral gene (110). Sequence comparison of NGF and proinsulin shows 30% and 52% identities in the regions of A and B chain of insulin, respectively. NGF isolated from mouse submaxillary gland contains two additional components--α and γ subunits (111,112). Nevertheless, only the β form (M_r 13,000) possesses nerve growth promoting and insulin-like activities (111). NGF plays an important role in the maintenancee and development of the sympathetic nervous system (113). In addition, it exerts the insulin-like activities with neurons of the sympathetic nervous system from embryo through adult life (113).

The third polypeptide showed to be insulin-related is relaxin. Relaxin is a hormone-like substance synthesized by the granulosa cells of corporsa lutea which acts on tissues of the pubic symphisis to aid the delivery of the fetus (114). It may play a more general role in tissue remodeling in the nonpregnant state. Again, isolated relaxin is a two-chain polypeptide joined by disulfide linkages (115,116), although some evidence suggested the existence of a single chain prorelaxin molecule (117). Primary structure comparison shows porcine relaxin and human IGF-1 share about 30% homology and both are related to insulin as well (118,119).

Mechanisms of Growth Stimulation and Oncogenesis

Normal diploid cells in cultures require serum to support efficient growth (74,120,121). In the absence of proper growth factors, cells arrested in the G1/G0 phase (7). Transformed cells have relaxed cell cycle control and may traverse the cell cycle in the absence of exogenous growth factors (122,123).

Recently, experimental data have been accumulated in favor of the notion that growth factor independence and autonomous growth of transformed cell might be due to a consitutive expression of any of the element along the normal mitogenic signal transmission pathway--the growth stimulatory factor itself, the membrane receptor that serves as a transducer of the extracellular signal, or the intracellular signal which ultimately leads to the initiation of DNA synthesis and cell proliferation. These constitutively expressed elements may themselves be an oncogene products or, alternatively, their expressions may be regulated by an oncogene product (124).

The first line of evidence in support of such a hypothesis was obtained by partial amino acid sequence analysis of separated PDGF polypeptide chains (A and B chains). It was shown that the B chain has a sequence virtually identical with part of the predicted sequence of the transforming protein $P28^{sis}$ (81, 82). $P28^{sis}$ is known to rapidly dimerize into a $P56^{sis}$ species. This product is then further processed by proteolytic cleavage. The apparently stable and end product is $P24^{sis}$, a polypeptide homodimer (81,125,126). The virtual identity between the PDGF-B chain and $P24^{sis}$ implies that SSV has acquired cellular sequences that are contained within the gene coding for the B chain (124).

By inference from the structural homology between the $P24^{sis}$ dimer and PDGF, it is assumed that the viral gene product acts as a PDGF agonist, i.e. it stimulates cell replication by interacting with the PDGF receptor. SSV-transformed cells do contain in their cytoplasm a growth factor activity and is cross-reactive to antibodies against PDGF (83). Whether the growth factor has to externalize and act analogously to PDGF by binding to the receptor at cell surface, or it can directly interact with the receptor within an intracellular compartment without being excreted is not known.

The synthesis of PDGF or a closely related polypeptide (see above) in human sarcoma and glioma cell cultures suggests that an abnormal expression of PDGF/c-sis gene may contribute to the transformation of these cells (75,76,79). This is also supported by the observation of high abundance of v-sis related m-RNA in human sarcoma and glioma cell lines (127).

Another example that the expression of a viral oncogene may turn on the production of a cellular growth factor has been found in cells transformed by murine sarcoma virus (MuSV) transformed cells (40). The transforming activity released from MuSV-transformed cells in most likely due the synergistic action of TGF-ß and TGF-α (41). It appears that TGF-α exerts part of its activity by binding to the EGF receptor.

The EGF agonistic activity of TGF-α is further supported by the observations that the transforming activity of TGF-α can be abolished by EGF receptor blocking antibodies (62); the TGF activated EGF receptor kinase activity was also blocked in a similar fashion. (128).

The production of TGF-α is not restricted in MuSV-transformed cell lines; rather, it is property of several transformed cell lines and also of normal tissues, including platelets (60). This suggests that most likely the TGFs are cellular gene products and are not encoded by viral genomes.

A structural relationship between growth factor receptors and oncogenes has been established by the finding that the EGF receptor has amino acid sequence homology with the transforming protein of avian erythroblastosis virus (AEV)--gp65^{erbB} (17). Sequence analysis revealed that the gp65^{erbB} corresponds to the internal and transmembrane domains of EGF receptor. This therefore implies the loss of the EGF binding domain may then be accompanied by a constitutive activation of the EGF receptor (17).

Again, inference from the structural homology between the p65^{erbB} and EGF receptor suggests that the viral gene product, independent of the presence of EGF may act as a constitutively activated receptor. While it is generally believed that autophosphorylation as well as tyrosyl phosphorylation of other cellular substrates seem to be integral activities of growth factor receptors (49,129,130), no kinase activity has been found associated with gp65^{erbB} (129). Whether this has a technical explanation or indicates another dimension of functional activity of gp65^{erbB} remains to be determined.

The mechanism by which the growth-factor stimulated mitogenic signals are transmitted from receptor and/or its neighboring cellular substrates and further into the cell is largely unknown. Recent studies on the c-myc oncogene may have the potential of enlightning this "black box". Addition of PDGF to fibroblasts or mitogen (concanavalin A or lipopolysaccharide) to lymphocytes results in an increase in the expression of c-myc in the stimulated cells (131). Since c-myc is identified as a nuclear protein (132,133), it is possible that c-myc may play a key regulatory role in the expression of a genetic program that controls cell proliferation. The myc gene, as transduced by a number of retroviruses, transforms a wide variety of cells from different histogenic origins (134). One explanation of this wide spectra of neoplasias with an abnormal expression of myc gene may be that several of the post-receptor signals ultimatlely converge at the regulation of the myc expression (124).

The conversion of a normal cell to a malignant one is a multi-step process (135-138). Recent transfection experiments have shown that while normal cells can not be fully transformed by a single oncogene, they can be transformed by co-transfecting along with another oncogene which belongs to a different complementary group (135,137). One of the best known examples is the myc and ras combination. When co-transfected into rat embryo fibroblasts (REF), dense foci of morphologically transformed cells were found. Acting together, myc and ras were able to do what neither could do on its own (138). Interestingly, while the revertants of virally transformed cells are resistant to be transformed by ras-related oncogenes (Ha-ras, Ki-ras, fes, src), they can be re-transformed by sis, mos, and fms oncogenes (139). This suggests that the activities of the ras-related oncogene products converge on a common target which is by-passed by the last three oncogene products (139). Again, this supports the idea that myc may regulate or itself represents one of these convergeing points. Therefore, signal transmission is mostly likely mediated by not a single, but a limited number of parallel post-receptor pathways, which may or may not be convergent, through which the proliferation is regulated.

In summary, the establishment of a direct mechanistic link between growth control and tumorigenesis has been a long-standing pipe-dream. But, in the past two years, such connection has been made. Several observations link different oncogenes to each of the level of action of growth factors. These include structural and functional similarities at the level of growth factor molecules, receptors of these growth factors, and probably the involvement of an oncogene product in the growth factor induced intracellular messenger system. Moreover, the enhanced expression of certain oncogenes has been found to cause endogenous production of growth factors, which then stimulates the producer cells in an autocrine manner. The general implication is that any regulatory element along this signal transmission pathway may have oncogenic properties if abnormally expressed or activated.

Recent Progresses in the Stuties of Growth Inhibitory Factors.

In contrast to the studies of growth stimulatory factors, much less progress has been made to establish a well defined system for the study of endogenous growth inhibitors that may function in cellular homeostasis. It has been hypothesized that a major control mechanism of cell renewal systems in vivo depends on certain negative feedback factors (chalones) that control the proliferation of cells. The existence of endogenous inhibitors of cell division was first suggested by the study of Bullough and Laurence on epidermal wound healing (for review, see reference (142-145). The distinguishing characteristics of these growth regulators are postulated to be: (a) total tissue specificity, (b) lack of species-specificity, and (c) reversibility.

A major difficulty in developing experimentally the chalone concept has been the formidable problem of isolation and chemical characterization of these molecules. Although growth inhibitory activity has been demonstrated in crude extracts from a number of tissues, much remains to be developed so that purification and molecular characterization of these factors can be accomplished. A key obstacle to be surmounted in any chalone purification program is the development of a reliable assay system, principally because of the technical problems associated with the extracts themselves. The preparations must not be inherently cytotoxic, or contaminated with bacterial products producing cytotoxic or cytostatic effects.

I will review recent developments in the isolation and characterization of negative regulators of cell growth (growth inhibitors), with particular emphasis on those molecules that have been purified and characterized (Table II).

Interferons. Interferons are a group of related glycoproteins (M_r 18,000-26,000) that induce an antiviral state in susceptible cells

Table 11. Properties of Some Negative Regulators of Cell Growth

Source	Nomenclature	Target (indicator cells)	Native Molecular Weight	SDS Gels (reducing conditions)	References
3T3 conditioned medium	FGR-s (13 K)	3T3 cells	13,000	13,000	166,167
various tissues, cells, and conditioned medium	transforming growth factor β (TGF-β)	NRK cells AKR-2B cells	25,000	12,500	41,154
human fibroblasts	interferon-β	HeLa	28,000-35,000	18,000	146
BSC-1 conditioned medium	BSC-1 growth inhibitor	CCL 64 mink lung	25,000	12,500	157,158
mouse embryo fibroblast conditioned medium	-	mouse embryo fibroblasts	10,000-15,000	14,000-11,000	170
rat liver	hepatocyte proliferation inhibitor	rat hepatocytes	26,000	26,000	162,163
bovine cerebral cortex cells	bovine glycopeptide inhibitor (BCSG)	mouse fibroblasts (3T3, LM), BHK-21	45,000	18,000-16,000	177
bovine mammary gland	-	Ehrlich ascites mammary cells	13,000	13,000	175

(146). Apart from their characteristic antiviral activity, interferons also inhibit significantly the proliferation of both normal and malignant cells, including fibroblasts, pluripotent hematopoietic stem cells and macrophage precursors (147-149). Specific cell surface receptors for interferons have been identified and the action of this family of proteins is believed to be hormone-like. Interferons have been studied extensively for biological activities other than growth inhibition. They have been reviewed elsewhere (146) and therefore, it will not be discussed further.

Hepatic proliferation inhibitor. An inhibitor of hepatocyte proliferation has been purified from rat liver (162,163). This hepatic proliferation inhibitor (HPI) yielded a polypeptide (M_r 26,000) in sodium dodecyl sulfate gels under reducing conditions. The isoelectric point of HPI is 4.65. This protein reversibly inhibited the proliferation of non-malignant rat liver cells in culture; it exerted no effect on the proliferation of hepatoma cells. Polyclonal rabbit antisera directed against HPI showed that parenchymal liver cells contained immune reactive HPI but endothelial and connective tissues did not (168). Little, if any, immunoreactive staining could be observed in hepatocellular carcinoma cells.

Transforming growth factors and growth inhibitor from conditioned medium of BSC-1 cells. Transforming growth factors (TGFs) are a family of polypeptides that reversibly induce non-neoplastic cells to express the transformed phenotype, as measured by loss of density-dependent inhibition of growth and acquisition of anchorage-independent growth (for review, see references (54,141). Initially discovered in the culture fluids from Moloney murine sarcoma virus transformed mouse cells (40), TGFs have also been isolated from conditioned medium or acid ethanol extracts of virally (17) or chemically (151) transformed cells as well as from human cancer cells (57).

Two distinct sets of TGFs have been purified to homogeneity. TGF-α is a single chain polypeptide (M_r 5,700) that shares sequence homology with epidermal growth factor (EGF) (see above). TGF-β is a distinct molecule consisting of two identical polypeptide chains (M_r 12,500) linked by disulfide bridges (41); the activity of native molecule (M_r 25,000) is destroyed upon reduction. TGF-β binds to its own unique cell surface receptors in responsive cells (152,153). TGF-β activity has also been found in serum-free conditioned medium of nontransformed mouse cell lines (154), as well as from diploid fibroblasts of human, mouse, and chicken embryos (155); the activity is enhanced by prior acidification, suggesting that the molecule may released in latent form. TGF-β has been purified from normal tissues such as human platelets, human placenta, and bovine kidney (156). Two non-neoplastic fibroblast indicator cell lines have been used for operational definition of TGF-β activity: normal rat kidney (NRK) cells required both TGF-β and either EGF or TGF-α to form colonies in soft agar (41); mouse embryo AKR-2 cells requier only TGF-β to grow under anchorage independent assay conditions (154).

Holley and co-workers have isolated a growth inhibitor from medium conditioned by African green monkey kidney cells (BSC-1) (157,158). This growth inhibitor yielded a native molecular weight of 25,000 and a polypeptide of M_r 12,500 after gel electrophoresis in the presence of sodium dodecyl sulfate and reducing agents. Recently, it has been shown that TGF-β and the growth inhibitor of BSC-1 cells have identical biological activities in : (a) stimulation of the growth of AKR-2B cells in soft agar; (b) inhibition of DNA synthesis in AKR-2B, BSC-1, AND CCL-64 (mink lung) cells; and (c) binding to TGF-β specific receptors on the cell surface (159). Therefore, TGF-β and the BSC-1 growth inhibitor are either identical or closely related molecules (Table 1). The results also suggest that the negative action of this TGF-β family of molecules in BSC-1 cultures is autocrine (141).

More recent data have shown that the response of cells to TGF-β is bifunctional (160). TGF-β inhibits the anchorage-dependent growth of many types of cells, including neoplastic and non-neoplastic cells of either fibroblastic or epithelial morphology. Under anchorage-independent conditions, TGF-β stimulates colony formation in NRK fibroblasts but inhibits growth of many human melanoma, lung carcinoma, and breast carcinoma cell lines over approximately the same concentration ranges (10-30 pM). Therefore, in certain instances, TGF-β stimulates anchorage-independent growth while in others it acts to inhibit that growth. Whereas EGF and TGF-β synergize to stimulate NRK growth in soft agar, their effects on A-549 human lung carcinoma cells are antagonistic.

The bifunctional response of cells to TGF-β cannot be ascribed to differences between non-neoplastic and neoplastic cell types nor to differences between fibroblastic and epithelial cell types. Rather, a bifunctional response to TGF-β has been shown to occur in a single cell type (c-myc-transfected Fisher rat 3T3 fibroblasts) under almost identical growth conditions and TGF-β concentration (160). In these cells, TGF-β synergizes with PDGF to stimulate colony formation but inhibits colony formation induced by EGF. These data indicate that the effects of the autocrine regulator TGF-β are not a function of the peptide itself but of the total set of growth factors (and their receptors) that is operating on a cell at a given time.

TGF-β now stands as a paradigm for several important features of growth regulation: (a) it is one of the first peptide growth inhibitors purified to homogeneity and its amino acid sequence has been determined; (b) it is a negative growth regulator that may function in the autocrine pathway and therefore neoplastic transformation may also result from failure to express or respond to specific growth inhibitory substances that are released by the cells to regulate their orderly growth; and (c) it carries bifunctional (stimulatory and inhibitory) growth regulatory activities. It is noteworthy that such a feature of peptide growth inhibitors has actually been predicted as recently as 1983 (161).

<u>Fibroblast growth regulator-soluble form.</u> A fibroblast growth regulator has been isolated from medium conditioned by exposure to density-inhibited mouse 3T3 cells. This growth inhibitory activity, termed FGR-s (Fibroblast Growth Regulator - soluble form), contained two polypeptides (M_r 10,000 and 13,000) (165,166). A monoclonal antibody (2A4), specifically bound the M_r 13,000 polypeptide of FGR-s and also neutralized the growth inhibitory activity in a concentration dependent a fashion (167). These results suggest that the M_r 13,000 polypeptide. designated FGR-s (13K), carries growth inhibitory activity. This polypeptide (pI ~10) chromatographed on gel filtration

as a M_r 13,000 species under native, non-reducing conditions.

Monoclonal antibody 2A4 also binds directly to live or unfixed 3T3 cells. The binding is saturable, suggesting that FGR-s (13K) or a cross-reactive precursor is present on the plasma membrane. This is consistent with the finding that radioactively-labeled preparations of FGR-s can bind to (or exchange with) components of the cell surface (168).

Particularly striking was the observation that addition of Antibody 2A4 to cultures of 3T3 cells in the absence of any exogenously added FGR-s enhanced the level of DNA synthesis (169). In contrast, addition of a control monoclonal antibody, which binds to 3T3 cells but is nonreactive with FGR-s, failed to yield the same effect. These results suggest that Antibody 2A4 may be neutralizing the activity of FGR-s molecules endogenous to the culture and reversing the effect of inhibition. Therefore, it is inferred that FGR-s (13K) may play a role in the normal mechanism of density dependent inhibition of growth in 3T3 cells. This negative regulation action of FGR-s (13K) in 3T3 cultures is autocrine.

Using procedures similar to our studies reported above, it has been shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose physico-chemical behavior closely parallels that of FGR-s. The molecular weights of the polypeptides in the active fractions were 11,000 and 14,000 (170). In this connection, it should be noted that both soluble as well as plasma membrane associated growth inhibitory fractions, derived from 3T3 cells and with properties similar to FGR-s, have been reported from several laboratories (171-174). The molecular properties of these active fractions and their relationship to FGR-s (13K) remain to be elucidated.

Other systems. Four additional growth inhibitory activities deserve mention simply because their characterizations have moved beyond mere demonstration of the phenomena of growth inhibition. They are: (a) a purified growth inhibitor (M_r 13,000) for Erlich Ascites mammary carcinoma cells from bovine mammary gland (175); (b) a partially purified glycopeptide preparation (M_r 18,000), derived from mouse and bovine cerebral cortex cells, that inhibits protein synthesis and cell growth of normal but not transformed cells (176,177); (c) a heparin-like molecule, produced by cultured endothelial cells, that inhibits the growth of smooth muscle cells (178,179); and (d) a lipid molecule on the plasma membrane of lymphoid cells that inhibits the growth of normal lymphocytes and lymphoid tumor cells (180,181).

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Chapter II

GROWTH CONTROL IN CULTURED 3T3 FIBROBLASTS

IV. Neutralization and Identification of a Growth Inhibitory Factor by a Monoclonal Antibody

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ABSTRACT

A fibroblast growth regulator, which inhibits the growth and division of proliferating fibroblasts, has been isolated from medium conditioned by exposure to density-inhibited mouse 3T3 cells. This partially purified preparation of the growth inhibitory activity, termed FGR-s, contained two major polypeptides (M_rs 10,000 and 13,000). Using FGR-s as the immunogen, we have carried out *in vitro* immunization of rat splenocytes and have generated hybridoma lines, each secreting an antibody directed against components of the FGR-s preparation. One such monoclonal antibody, designated 2A4, specifically bound the M_r = 13,00 polypeptide of FGR-s. Antibody 2A4 also neutralized the growth inhibitory effect of FGR-s in a concentration dependent fashion. These results strongly suggest that the M_r = 13,000 polypeptide carries growth inhibitory activity.

The mouse fibroblast line 3T3 exhibits a characteristic form of growth control *in vitro* in that the cells reach only a very low saturation density and can remain for long periods of time in a viable but nonproliferating state (1,2). Although the mechanisms responsible for this phenomenon are not completely understood, a number of lines of evidence suggest that inhibitory factors released into the medium by the 3T3 cells themselves may be responsible for at least part of the observed suppression of cell division (3).

In previous studies, we demonstrated that medium conditioned by exposure to cultures of density-inhibited 3T3 cells contained a growth inhibitory activity that acted on sparse, proliferating cultures of the same cell line (4). This inhibitory activity was fractionated, yielding one preparation that had the following key properties (5,6): (a) it consists of two polypeptide chains (M_rs 10,000 and 13,000); (b) it is an endogenous product, elaborated by the 3T3 cells and released into the medium; (c) it is not cytotoxic and its effects on target cells are reversible; and (d) it interacts directly with the target cells. We have designated this fraction FGR-s, which stands for Fibroblast Growth Regulator that is secreted or shed into the medium in a soluble form.

Although the evidence suggested that one or both polypeptides observed in the FGR-s fraction were responsible for the growth inhibitory activity, we could not make a definite assignment of the biological activity. One approach to this problem is to raise monoclonal antibodies that will bind and/or neutralize the growth inhibitory activity. In the present communication, we report the generation and characterization of one such monoclonal antibody, designated 2A4, which will counteract the activity of FGR-s. The use of Antibody 2A4 has allowed us to conclude that the $M_r = 13,000$ polypeptide is responsible for at least part of the biological activity of FGR-s.

MATERIALS AND METHODS

Cultures of 3T3 Cells and Production of FGR-s

Mouse 3T3 fibroblasts were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco Modified Eagle's Medium (DME) (K.C. Biological, Lenexa, KA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% calf serum (Microbiological Associates, Walkersville, MD). The 3T3 cells were passaged before the cell reached a confluent state (4). The growth medium was first removed, and the cells were washed and then incubated in PBS with 0.25% trypsin (Nutritional Biochemicals, Cleveland, OH) and 4×10^{-4} M Versene at 37° for ten minutes. The cells were dislodged from the growth surface, transferred and centrifuged at 1320 x g for three minutes. The trypsin solution was removed and the cells were resuspended in DME containing 10% calf serum and seeded at the desired density.

The procedures for the production and isolation of FGR-s have been described (5). Briefly, 3T3 cells were seeded at an initial density of 2×10^4 cells/cm^2 and allowed to grow to a confluent monolayer. The growth medium was then removed and the cells were washed twice with serum free DME. After washing, the cells were incubated in DME (10 ml per 150 cm^2 of growth surface) for 20-24 hours. The medium from this incubation was collected and centrifuged at 1470 x g for ten minutes to remove cellular debris and particulate material. The supernatant, hereafter designated serum-free conditioned medium, was the starting material for fractionation. To prepare FGR-s labeled with [^{35}S]methionine, the cells were cultured in DME containing 3 μg/ml unlabeled methionine (one tenth of the concentration normally found in DME) and 10 µCi/ml of [^{35}S]methionine (1014 Ci/mmol, New England Nuclear, Boston, MA).

Solid ammonium sulfate was added to serum-free conditioned medium to a saturation of 80% at room temperature. All subsequent operations were performed at 4°. The ammonium sulfate precipitated mixture was centrifuged at 12,400 x g for 15 minutes and the supernatant was decanted. The precipitate collected from 800 ml serum-free conditioned medium was resuspended in 2 ml of DME and fractionated on a column (1.4 x 110 cm) of Sephadex G-50 equilibrated with DME. FGR-s represents the material eluting from the Sephadex G-50 column at a position corresponding to a molecular weight of 10,000-15,000. This region contained material previously shown to be enriched in specific biological activity (5). This preparation was used for the immunization of rat spleen cultures for the generation of antibody secreting cells as well as for the screening of hybridoma clones.

Immunization of Rat Splenocytes with FGR-s

The *in vitro* immunization procedure (7), outlined Fig. 1, was used. Briefly, thymocytes from a 21-day old female rat were isolated and used to condition the medium (5 x 10^6 cells/ml; 40 ml of DME containing 20% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM glutamine) for 48 hours at 37°. The conditioned medium was used for the culture of rat spleen cells in the presence of the immunogen, FGR-s. Spleen cells from a female rat were suspended at a density of 1.0 x 10^7 cells/ml in DME containing 2 mM glutamine and 50 µM mercaptoethanol. The spleen cell suspension (4 ml) was first mixed with 1 ml of

In Vitro Immunization

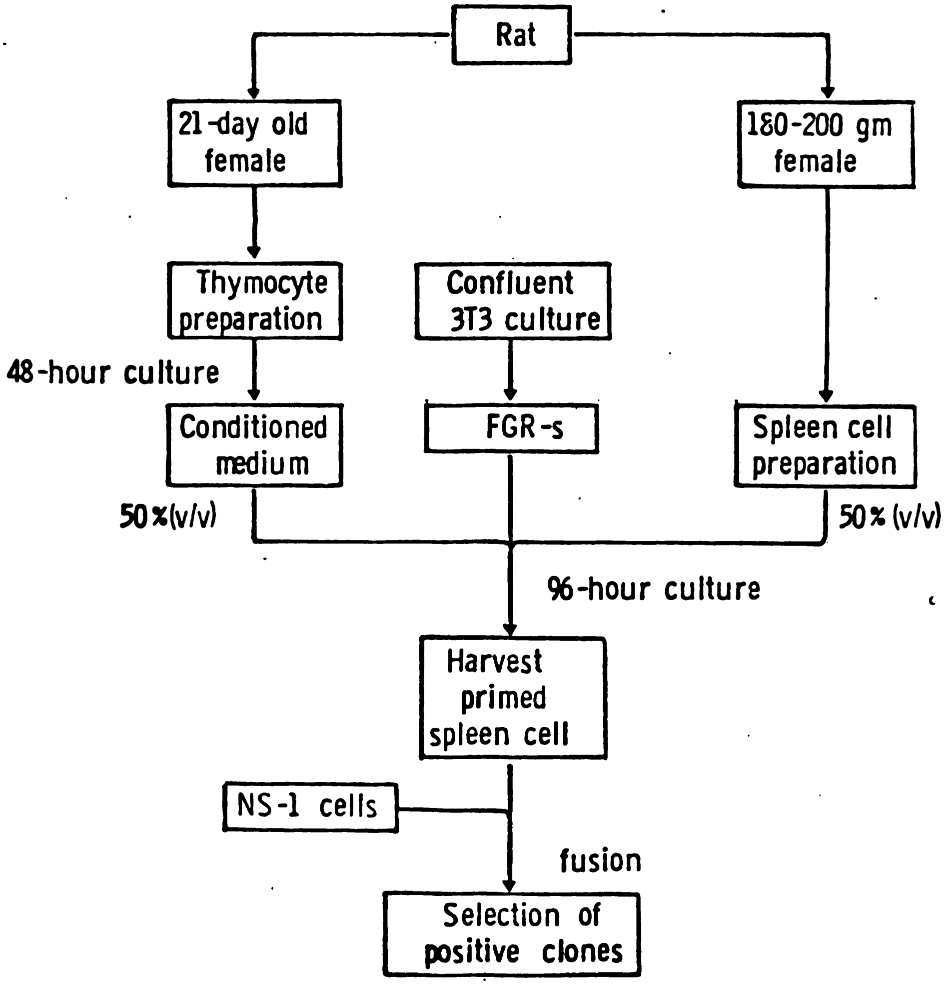


Figure 1. Outline of the procedure used for the generation of monoclonal antibodies directed against components of FGR-s using the *in vitro* immunization procedure.

FGR-s (100 ng/ml) and incubated at 37°C. After 45 minutes, 5 ml of thymocyte conditioned medium (resupplemented with 2 mM glutamine and 50 µM mercaptoethanol), 0.5 ml fetal bovine serum, and 0.5 ml horse serum were then added. The mixture was then cultured at 37°C for 96 hours. The spleen cells were then harvested and fused with the mouse myeloma cell line, NS-1Ag4/1 (Salk Institute), using polyethylene glycol 1500 (8). The ratio of immune spleen cells to myeloma cells used in the fusion was 10:1. Hybridoma cultures were selected and cultured as described (8).

In Vivo Immunization of Rats with 3T3 Cells - We have also carried out immunization of rats with 3T3 cells to generate monoclonal antibodies that bind to whole 3T3 cells but not specifically directed against the FGR-s fraction. Confluent monolayers of 3T3 cells (4 x 10^4 cells/cm^2) were scraped with a rubber policeman washed with 10 ml of phosphate buffered saline (PBS). The cells were resuspended in PBS at a density of 1.0 x 10^7 cells/ml and emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). Female Sprague- Dawley rats (200 gm, Charles River Co.) were immunized intraperitoneally with 1 ml aliquots of the emulsion. The rats were given booster injections of similar doses in Freund's incomplete adjuvant at days 8 and 31. When the serum from an immunized rat was assayed as positive in the primary screening assay, the rat was boosted again and sacrificed 7 days later. The spleen cells were prepared and were fused with mouse myeloma NS-1 cells as described above (8).

Primary Screening Assay (see Fig. 2) - Antisera from the immunized rats or supernatants from selected hybridoma cultures were tested by this assay. 3T3 cells (3 x 10^5 cells/well) were seeded in 96-well polyvinyl plates (Costar, Cambridge, MA) and cultured at 37°C, for 24 hours. All further steps were done at room temperature. The attached 3T3 cells were fixed with 0.25% (w/v) glutaraldehyde in PBS for 10 minutes. The cells were then washed 3 times in PBS, and bovine serum albumin (3% (w/v), BSA) in PBS was then added and incubated for one hour and then washed as above. Antisera or supernatants from hybridoma cultures were added, incubated for an hour. Finally, I^{125}-rabbit-anti-rat IgG (5 x 10^8 cpm/mg; 1 x 10^5 cpm; 50 μl) was added and incubated for an hour. After unbound radioactivity was removed by washing, the polyvinyl plates were then cut into individual wells and the amount of radioactivity in each well was measured using a LKB RIA gamma counter.

Screening Assays for Hybridoma Cultures Producing Antibodies Directed Against FGR-s - A modification of the primary screening assay was developed to detect hybridoma clones secreting antibodies directed specifically against a partially purified protein fraction (e.g. FGR-s). This assay required the efficient adsorption of the specific antigen fraction onto solid surfaces to minimize the amount of antigen used during the screening process. Moreover, because of the limited amount of material available, this screening assay was used only on those hybridoma clones that showed positive reaction in the primary screening assay, thus indicating that these clones may be "putative positives" in terms of clones that secrete monoclonal antibodies directed against our antigen of interest, FGR-s.

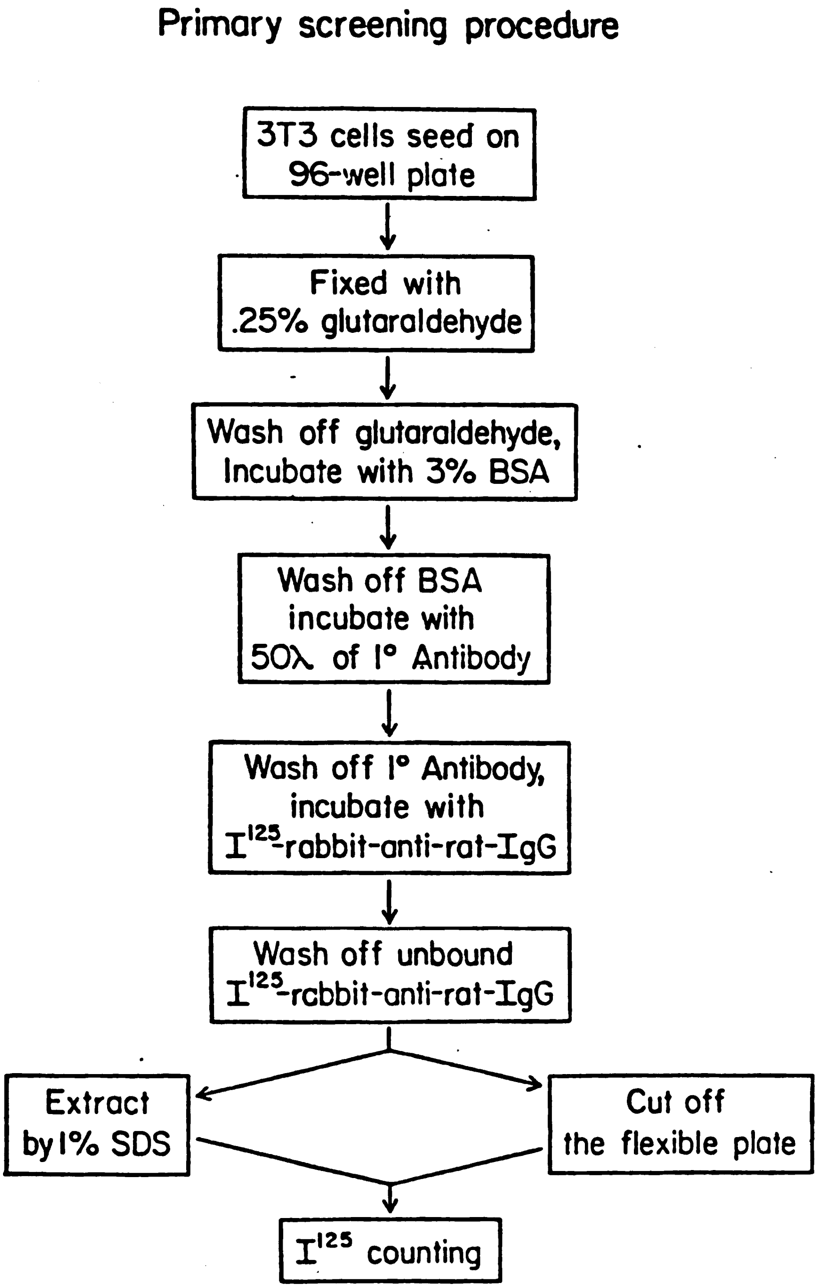


Figure 2. A schematic diagram outlining the primary screening assay. Primary antibody is either serum or supernatant from hybridoma cultures.

Unlabeled FGR-s was bound to individual wells of Immulon-2 microelisa plates (Dynatech, Alexandria, VA). The wells were washed three times in phosphate-buffered saline (PBS) and bovine serum albumin (3% w/v) in PBS was then added and incubated for one hour. The wells were then washed as above. Supernatants (50 µl) from hybridoma cultures or immunoglobulin purified from such supernatants were added and incubated for one hour. Finally, ^{125}I-labeled rabbit antibodies directed against rat immunoglobulin (5×10^8 cpm/mg; 1×10^5 cpm; 50 µl) was added and incubated for one hour. After unbound radioactivity was removed by washing, the plates were cut into individual wells and the amount of radioactivity in each well was measured in a gamma counter.

Classification of Antibody Secreted by Hybridoma Clones - The type of immunoglobulin (IgG, IgM, etc.) secreted by the selected hybridoma clones was identified by immunoprecipitation. Rabbit-anti-rat IgG (Sigma, St. Louis, MO) was incubated overnight with an equal volume of a 10% (v/v) fixed S. aureus cell suspension at 4°C. The cells were pelleted by centrifugation (1500 x g, 10 minutes) and then resuspended to a concentration of 10% (v/v) with 3% BSA (w/v) in PBS and incubated for an hour at room temperature. The suspension was then washed 3 times by centrifugation and resuspension with Tris-HCl buffer (0.1 M, pH 7.4, 0.05% Nonidet P-40). Equal amount (50 µl) of ^{35}S-methionine labelled (100 µCi/ml, 1000 Ci/mmole, 18 hours) hybridoma supernatant and S. aureus suspension were added together and incubated at 4°C for an hour. The suspension was then washed with Tris-HCl buffer and subjected to analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).

Assay of Cell Proliferation - Target cells were seeded in DME-5% calf serum at a density of 5 x 10^3 cells/cm^2 in 96-well culture dishes (Costar). After 48 hours, 100 μl of the test sample was added to the cultures. This 100 μl of test sample contained: (a) both FGR-s (50 μl) and Antibody 2A4 (50 μl); or (b) FGR-s (50 μl) and DME (50 μl); or (c) Antibody 2A4 (50 μl) and DME (50 μl); or (d) only DME (100 μl). After incubation for 24 hours, the cells were pulsed with 1 μCi/well of [^{3}H]thymidine (1.9 Ci/mmol, Schwarz-Mann) for 3 hours at 37°. The cells were harvested for quantitation of [^{3}H]thymidine incorporation as previously described (5).

Affinity Chromatography Procedures - Rabbit antibodies directd against rat immunoglobulin were coupled to Affi-Gel 10 (Bio Rad) using the procedure of Ikeda and Steiner (10). Supernatant from the hybridoma clone 2A4 was passed through the column (12 x 1 cm) three times. Material bound non-specifically was removed by washing with 0.1 M phosphate buffer (pH 8.0). The bound material was eluted with 0.1 M citrate buffer (pH 3.0), dialyzed overnight against PBS with three changes of buffer, and subjected to polyacrylamide gel electrophoresis analysis. The isolated Antibody 2A4 was stored at -20°C.

In a similar fashion, Antibody 2A4 was coupled to Affi-Gel 10 as described above. This affinity column (4 x 0.6 cm) was used to fractionate FGR-s preparations to determine the molecular identity of the antigenic target for Antibody 2A4.

Polyacrylamide Gel Electrophoresis - Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to the procedure of

Laemmli (11), using 10% or 16% acrylamide running gel and 5% acrylamide stacking gel. β-Mercaptoethanol (4%) was included in the sample buffer. The gels were fixed and then stained with Coomassie Brilliant Blue. With radioactive samples, the gel was subjected to fluorographic treatment as described by Bonner and Laskey (12), using Kodak X-Omat R (XAR-5) film.

RESULTS

Monoclonal Antibodies that Bind to 3T3 Cells

Rats were immunized in vivo with 3T3 cells and their sera were tested for antibodies directed against the immunogen. Figure 3 shows the results of the primary screening assay using the serum collected from the rat whose spleen cells were subsequently used for fusion. Serial dilutions of the immune serum (1:128 to 1:2048) resulted in a linear decrease of the binding of ^{125}I- labelled antibody. On the other hand, higher concentration of immune serum (1:64 and 1:32 dilutions) also showed lower binding of second antibody. The reason for this decrease at high concentrations is not known. Nonetheless, it is obvious that the primary screening assay can clearly distinguish normal serum (preimmune) from serum containing antibodies directed against 3T3 cells.

Hybridomas which produce antibodies reacting with fixed 3T3 cells were detected by the primary screening assay and were cloned in soft agar. The clones and their secreted products were numbered according to the sequence of establishment of the clones. Supernatants from these clones were tested using primary screening assay; the results are shown in Fig. 4. These clones exhibited 1.7 to 4.4 fold greater binding activity as compared to that seen using the supernatant from the parental myeloma NS-1 cell line.

Monoclonal Antibodies Directed Against FGR-s

Rat spleen cells were immunized in vitro with FGR-s as outlined in Fig. 1. After 96 hours of culture, the rat lymphocytes were fused with

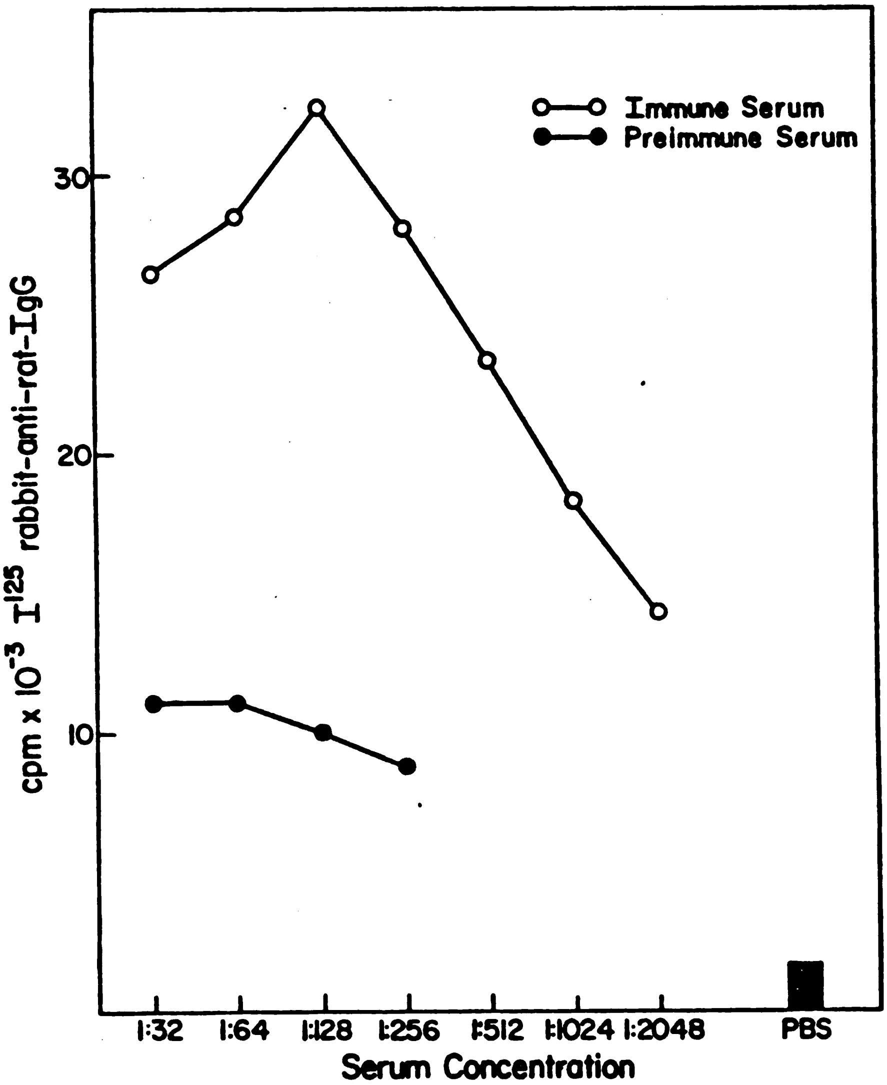


Figure 3. Effect of serial dilution of the response of immune serum in the primary screening assay. Immune and preimmune serum were serially diluted in PBS as indicated and tested in the primary screening assay.

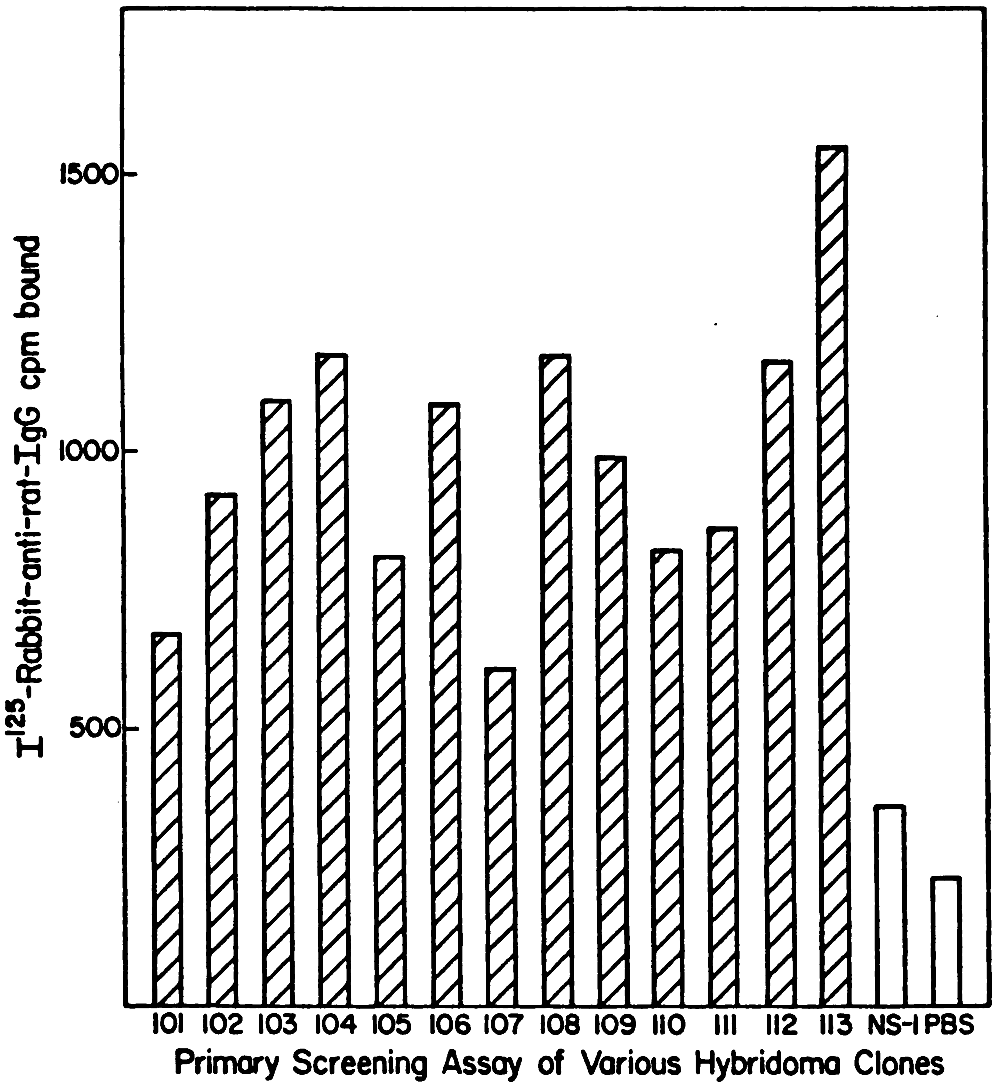


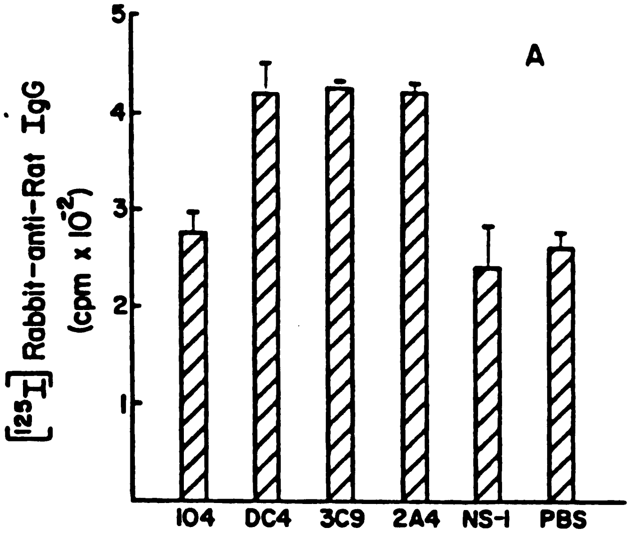
Figure 4. Summary of the results of the primary screening assay of clones 101 to 113 derived from in vivo immunization of rats with whole 3T3 cells. The supernatant from clones were tested as described in Materials and Methods. Supernatant from NS-1 culture or PBS were used as controls.

NS-1 myeloma cells. Hybridoma cultures which produced antibodies reacting with the FGR-s fraction were detected by the binding of rat immunoglobulin in the culture supernatants to FGR-s adsorbed onto Immulon-2 plates. Positive cultures were cloned and their secreted products were tested for binding to both FGR-s as well as to whole 3T3 cells (Fig. 5).

The supernatants from three hybridoma lines, designated DC4, 3C9, and 2A4, showed positive reactions when assayed with FGR-s on Immulon-2 plates. Compared to the supernatant of NS-1 myeloma cultures, each of the hybridoma supernatants exhibited 1.5-1.7-fold more binding (Fig. 5A). This binding was specific. When bovine serum albumin was substituted for FGR-s on the Immulon-2 plates, the binding of the supernatants from DC4, 3C9, 2A4 and NS-1 cells were all the same. The supernatants of clones 2A4 and 3C9 were also positive when assayed with whole 3T3 cells fixed to the plates, exhibiting 1.4-1.6 fold more binding than the corresponding supernatant from NS-1 cells (Fig. 5B).

Quite the opposite results were obtained with hybridoma clones which were derived from rats immunized in vivo with whole 3T3 cells and screened and selected on the basis of binding to whole 3T3 cells. A representative clone, designated 104 (see Fig. 4), is used here for illustrative purposes. Clone 104 showed strong reaction (2.4-fold) when assayed on whole 3T3 cells (Fig. 5B) but negligible reaction when assayed on FGR-s (Fig. 5A). These results suggest that the products of clones 2A4 and 3C9 were monoclonal antibodies directed against some component of FGR-s, which in turn is a constituent of whole 3T3 cells.

Figure 5. Summary of the results of screening assays carried out using (A) FGR-s and (B) whole 3T3 cells as targets. FGR-s or 3T3 cells were immobilized by attachment to microtitre plates as described in the text. The supernatants from clones derived from *in vitro* immunization of FGR-s (DC4, 3C9, and 2A4) and from *in vivo* immunization of whole 3T3 cells (104) were tested. The supernatant from the parent myeloma line, NS-1, was used as a control.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4
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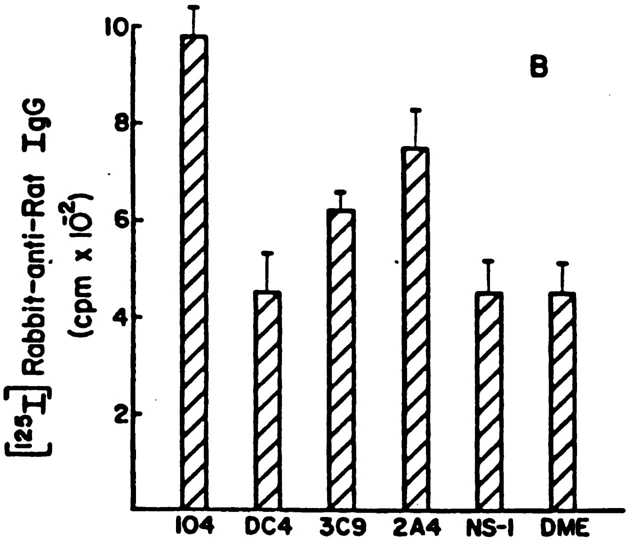
2A4
NS-1
PBS


B
[125I] Rabbit-anti-Rat IgG
(cpm x 10-2)
10
8
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4
2
104
DC4
3C9
2A4
NS-1
DME

Isolation and Characterization of Antibody 2A4

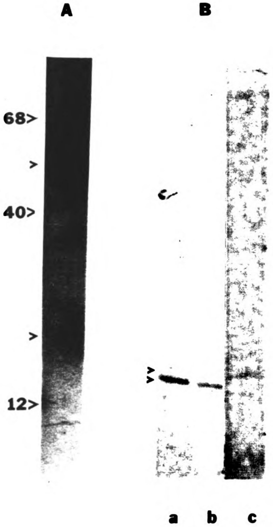
The class of antibody secreted by clone 2A4 and clone 104 was determined by immunoprecipitation of the supernatant from ^{35}S-methionine labelled culture of individual clones. *S. aureus* were precoated with rabbit-anti-rat IgG and then incubated with ^{35}S-labelled supernatant from clone 2A4. Gel electrophoretic analysis of the bound material revealed two distinct polypeptides of M_r 55,000 and 22,000; this suggests clone 2A4 synthesized and secreted immunoglobulin G. Similar results were obtained with clone 104. No detectable polypeptide was identified with the ^{35}S-methionine labelled supernatant from the parental myeloma NS-1 cells.

The supernatant from hybridoma clone 2A4 was fractionated on an affinity column of Affi-Gel 10 covalently derivatized with rabbit antibodies directed against rat immunoglobulin. Material bound to the column was eluted with 0.1 M citrate buffer (pH 3.0) and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Two polypeptides, corresponding to the heavy (M_r 55,000) and light (M_r 23,000) chains of immunoglobulin, were observed (Fig. 6A). These results establish that the product of clone 2A4 was a rat IgG molecule. All subsequent experiments were performed with the purified IgG molecule, designated Antibody 2A4.

Identification of the Target of Antibody 2A4

When a [^{35}S]methionine-labeled preparation of FGR-s was fractionated on a column of Affi-Gel-10 derivatized with Antibody 2A4, approximately 90% of the radioactivity failed to bind to the column (Component A, Fig. 7). The remainder of the radioactivity was bound

Figure 6. (A) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the immunoglobulin derived from hybridoma clone 2A4. The acrylamide composition of the gel was 10% and the gel was stained with Coomassie Blue. The arrows indicate the positions of migraton of molecular weight markers and authentic immunoglobulin heavy and light chains. (B) Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of [^{35}S]methionine-labeled FGR-s fraction and components derived from chromatography of FGR-s over an affinity column containing Antibody 2A4. The acrylamide composition of the gel was 16% and the gel was subjected to fluorography for 38 days (600 cpm per lane). Lane a: FGR-s; lane b: Component A, Fig. 7; lane c: Component B, Fig. 7. The arrows indicate the positions of migration of M_r = 13,000 and M_r = 10,000 polypeptides.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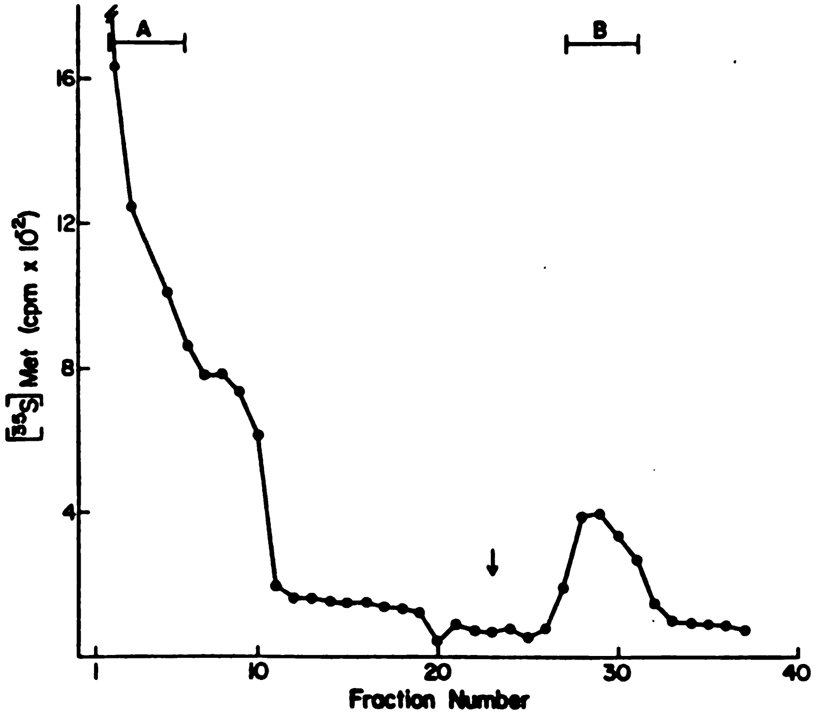

Figure 7. Chromatography of [^{35}S]methionine-labeled FGR-s on an affinity column (4 x 0.6 cm) of Antibody 2A4 coupled to Affi-Gel 10 equilibrated with PBS. At the point indicated by the arrow, 0.1 M citrate buffer (pH 3.0) was used to elute the bound material.

and could be eluted with citrate (Component B, Fig. 7). Polyacrylamide gel electrophoretic analysis of Component A (Fig. 7) showed one predominant polypeptide (M_r 10,000). The material bound by Antibody 2A4 (Component B, Fig. 7) also showed only one polypeptide band, corresponding to a molecular weight of 13,000 (Fig. 6B). Together, the gel patterns of Components A and B (Fig. 7) accounted for the two major polypeptides seen in the FGR-s fraction (Fig. 6B). These results indicate that Antibody 2A4 is specifically directed against the M_r = 13,000 polypeptide in the FGR-s fraction.

Effect of Antibody 2A4 on the Growth Inhibitory Activity of FGR-s

To test the possibility that Antibody 2A4 can neutralize the growth inhibitory activity of FGR-s, the effect of the inhibitor preparation on [^{3}H]thymidine incorporation in target 3T3 cells was assayed in the presence and absence of the purified antibody. We had previously demonstrated that the growth inhibitory effect of FGR-s on 3T3 cells was dependent on the concentration of the ligand added (5). In the present assay, 60% inhibition was obtained with 20 µl of the FGR-s preparation (Table 1). When the effect of FGR-s was assayed in the presence of Antibody 2A4 (25 ng/ml), however, the inhibition was completely abrogated. The level of DNA synthesis in this case was the same as that of control cultures, without any FGR-s or Antibody 2A4. Similarly, the addition of 25 ng/ml of Antibody 2A4 reduced the inhibitory effect of 50 µl of FGR-s from 80% to 35%. Thus, when the effect of FGR-s was assayed in the presence of Antibody 2A4, there was always a higher level of [^{3}H]thymidine incorporation (i.e. a reduced level of growth inhibition).

Table 1. The effect of FGR-s and Antibody 2A4 on DNA synthesis in 3T3 fibroblasts.*

Antibody Treatment	FGR-s added (μl)		
	0	20	50
None	5219 ± 934	2248 ± 404	1085 ± 174
2A4 (25 ng/ml)	11096 ± 1182	5430 ± 619	3409 ± 307
104 (25 ng/ml)	4599 ± 284	2783 ± 325	1143 ± 113

*The data are expressed in terms of [^{3}H]thymidine incorporation (cpm) in target cells and represent averages of triplicate determinations ± standard error of the mean.

Particularly striking was the observation that Antibody 2A4 (25 ng/ml) also increased the level of DNA synthesis in 3T3 cultures in the absence of any exogenously-added FGR-s (Table 1). Similarly, when a small amount of FGR-s was used, resulting in 25% inhibition, the addition of Antibody 2A4 raised the level of DNA synthesis even beyond that of control cultures, without any FGR-s or Antibody 2A4 (data not shown). Therefore, over the entire range of FGR-s concentration tested, the addition of Antibody 2A4 resulted in a higher level of DNA synthesis.

These effects of Antibody 2A4, on DNA synthesis of 3T3 cells and on the effect of FGR-s, were specific. Antibody 104, which was not reactive with FGR-s polypeptides (Fig. 5), failed to yield the same effects (Table 1). These observations suggest that the results obtained with Antibody 2A4 are most probably not due a growth factor contaminating the immunoglobulin fraction. This conclusion is further supported by experiments that showed the same effects of Antibody 2A4 when the assays were carried out in the presence of freshly added calf serum (5%).

The present results on the neutralization of growth inhibitory activity of FGR-s by Antibody 2A4 (Table 1), coupled with the fact that this antibody specifically recognizes the $M_r = 13,000$ polypeptide (Fig. 6B), indicates that this polypeptide carries growth inhibitory activity.

DISCUSSION

In previous studies, we demonstrated that medium conditioned by exposure to cultures of density-inhibited 3T3 fibroblasts contained a growth inhibitory activity that acted on sparse, proliferating cultures of the same cells (4). This activity was partially purified, resulting in a fraction called FGR-s which contained two polypeptides (M_rs 10,000 and 13,000) (5). Using the FGR-s fraction as immunogen, we have generated a monoclonal antibody (2A4) which has allowed us to demonstrate that a growth inhibitory activity is associated with the M_r = 13,000 polypeptide. This conclusion is derived from two key observations in the present study: (a) affinity chromatography of ^{35}S-labeled FGR-s on a column containing Antibody 2A4 resulted in the specific binding of the M_r = 13,000 polypeptide to the column; (b) when the biological activity of FGR-s was assayed in the presence of Antibody 2A4, there was a reduction in the level of growth inhibition.

We had previously shown that ^{35}S-labeled FGR-s preparation bound specifically to the target 3T3 cells (6). When these cells were washed to remove non-bound radioactivity and extraced with mild acid, only the polypeptide with M_r = 10,000 was recovered in the acid fraction. The possibility was raised that the M_r = 13,000 polypeptide was internalized and/or degraded during the course of the incubation. We now find, however, that Antibody 2A4, which specifically recognizes the M_r = 13,000 protein, binds to the surface of 3T3 cells. Therefore, the M_r = 13,000 protein or a higher molecular weight precursor, is present on the plasma membrane.

The assignment of biological activity to the M_r = 13,000 polypeptide does not exclude the possibility that other components of the FGR-s fraction (e.g., M_r = 10,000 polypeptide) may also be biologically active. The use of Antibody 2A4 affinity column to fractionate FGR-s, yielding a preparation containing only the M_r = 10,000 polypeptide (Component A, Fig. 7) and a preparation containing only the M_r = 13,000 polypeptide (Component B, Fig. 7), has been carried out only at the analytical level. When this fractionation is achieved at the preparative scale, we would then be able to assay each individual polypeptide for biological activity. It is also possible that both polypeptides are necessary for activity so that reconstitution experiments are required.

In any case, if the M_r = 13,000 polypeptide (and other components of FGR-s) plays a physiologically significant role in density-dependent inhibition of growth, one would expect that addition of Antibody 2A4 to dense cultures of 3T3 cells should at least partially neutralize the activity of endogenous FGR-s molecules in the culture and reverse the effect of density inhibition. Our observations lend support to this prediction. The addition of Antibody 2A4 in the absence of exogenously-added FGR-s increases DNA synthesis in the 3T3 cultures. In contrast, the addition of Antibody 104, which is not reactive with FGR-s, failed to yield the same effect.

The successful generation of a monoclonal antibody directed against an active polypeptide of the FGR-s fraction will allow us to (a) isolate the regulatory polypeptide by affinity chromatography; (b) compare the amount of this polypeptide in normal versus transformed cells, fibroblasts versus other cell types, cells at different densities or at different stages of the cell cycle; (c) localize and trace the fate of this polypeptide in target cells; (d) search for precursor or other forms of the growth regulator; and (e) search for and identify the receptor for this polypeptide, which binds to the target cells.

In addition, it would be of obvious interest to establish the relationship between the M_r = 13,000 polypeptide in FGR-s and similar growth inhibitory activities reported in other systems. These include: (a) the membrane-bound growth inhibitory activities of 3T3 cells reported by Wittenberger and Glaser (13), Liberman et al. (14), Peterson et al. (15), Peterson and Lerch (16), Natraj and Datta (17), and Datta and Natraj (18); (b) the highly purified growth inhibitory factor from medium of BSC-1 cells (19,20); (c) the hepatic proliferation inhibitor (M_r 26,000; pI 4.65) isolated from normal rat livers (21, 22); and (d) the growth inhibitory glycopeptides identified and partially purified from mouse and bovine cerebral cortex cells (23,24). More recently, it has been shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose physico-chemical behavior closely parallels that of FGR-s (25) and that a growth inhibitor for Ehrlich ascites mammary carcinoma cells can be isolated from the bovine mammary gland (26). The use of Antibody 2A4 in biological activity assays and in immunoprecipitation or immunoblotting experiments with active preparations of the growth inhibitory activities from the various sources could facilitate the identification of the relevant polypeptide(s) and reveal any structural similarities with the FGR-s polypeptide.

The successful generation of a monoclonal antibody directed against a component of the FGR-s fraction exemplifies the powerful utility of the _in vitro_ immunization procedure as well as the usefulness of Immulon-2 for adsorption of proteins for screening assays. We estimate that the concentration of FGR-s used in the _in vitro_ immunization procedure was 10 ng/ml. Luben and Mohler (7) and Pardue et al. (27) have reported successful _in vitro_ immunization using similar levels of antigen. For the screening of hybridoma cultures producing antibodies directed against FGR-s, we estimate that approximately 10 ng of FGR-s protein was adsorbed onto individual wells of the Immulon-2 plate. This represented about 36% of the FGR-s protein added to each well. Clearly, hybridomas can be generated by _in vitro_ immunization using minute amounts of immunogen.

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Chapter III

GROWTH CONTROL IN CULTURED 3T3 FIBROBLASTS

V. Purification of a M_r 13,000 Polypeptide Responsible for Growth Inhibitory Activity

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SUMMARY

A fibroblast growth regulator, which inhibits the growth and division of proliferating fibroblasts, has been isolated from medium conditioned by exposure to density-inhibited mouse 3T3 cells. This factor, termed FGR-s (13 K), yielded a single polypeptide ($M_r = 13{,}000$) when analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate under both reducing and non-reducing conditions. The dose-response curve of growth inhibition by FGR-s (13 K) showed that 50% inhibition of 3T3 cell proliferation was achieved at a concentration of 3 ng/ml, corresponding to 2.3×10^{-10} M. The inhibition was reversible. These results indicate that we have purified a growth regulatory factor that acts to inhibit the proliferation of cells in an autocrine pathway.

The mouse 3T3 fibroblast line exhibits a form of growth control *in vitro* in that the cells reach only a low saturation density and can remain for long periods of time in a viable, monolayer state (1,2). Treatment of sparse, proliferating cultures of 3T3 cells with medium conditioned by exposure to density inhibited 3T3 cultures resulted in an inhibition of growth and division in the target cells when compared with similar treatment with unconditioned medium (3). This inhibitory activity was fractionated, yielding one preparation that exhibited reversible inhibition of growth and direct interactions with target 3T3 cells (4,5). This fraction, designated Fibroblast Growth Regulator - soluble form (FGR-s)[1], contained two major polypeptides (M_rs 10,000 and 13,000) (4,6). Using similar procedures, Wells and Mallucci have shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose polypeptide composition and physicochemical behavior closely parallel those of FGR-s (7).

More recently, we have generated and characterized a monoclonal antibody (Antibody 2A4) that neutralized the activity of FGR-s (8). This monoclonal antibody specifically bound the M_r 13,000 polypeptide, hereafter designated as FGR-s (13 K). On the basis of these data, we concluded that FGR-s (13 K) is responsible for at least part of the observed growth inhibitory activity. This assignment of biological activity to FGR-s (13 K) does not exclude the possibility that other components of the FGR-s fraction (e.g. M_r 10,000 polypeptide) may also be biologically active. Moreover, we could not ascertain whether FGR-s (13 K) was active independent of the presence of other polypeptides (8,9). In the present communication, we report the purification to apparent homogeneity of FGR-s (13 K) on a preparative scale. This has allowed us to assay the individual polypeptide for biological activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of FGR-s - Swiss 3T3 cells (American Type Culture Collection, CCL 92) were grown at 37° in Dulbecco modified Eagle's medium (DME, K.C. Biological) containing 10% calf serum (Microbiological Associates). The detailed protocol for the preparation of FGR-s has been described (3,4). Briefly, confluent monolayers of 3T3 cells were washed twice with DME; fresh, serum-free DME was then added to the cultures (10 ml/150 cm^2 of growth area). After overnight incubation, the medium was collected as serum-free conditioned medium. This medium was centrifuged at 1470 x g for 10 minutes and the supernatant was subjected to ammonium sulfate precipitation (80% of saturation at room temperature). The precipitate was redissolved in 5 mM Tris, pH 8.0 (2.5 ml of buffer per liter of conditioned medium) and fractionated by Sephadex G-50 chromatography (1.4 x 110 cm) in the same buffer. The material eluting at a position corresponding to polypeptides of molecular weight 10,000 to 15,000 was pooled as the FGR-s fraction.

Preparation of FGR-s labeled with [^{35}S]methionine was carried out as described, using 100 µCi of radioactive methionine per ml (Amersham, 1014 Ci/mmole) and DME containing unlabeled methionine at 3 µg/ml (one tenth of the concentration normally found in DME) (4).

Ion-exchange Chromatography on DEAE-cellulose - A column (0.8 x 2 cm) of DEAE-cellulose (Pharmacia), was equilibrated with 5 mM Tris, pH 8.0. The pooled material corresponding to the FGR-s fraction of the Sephadex G-50 column (4) was applied to the ion-exchange column. After washing with starting buffer, a gradient (0 to 0.5 M NaCl in 100 ml of 5 mM Tris, pH 8.0) was used to develop the column. Fractions of 1.7 ml were collected.

Assays of DNA Synthesis - Target cells used to test the growth inhibitory activity were routinely seeded at an initial density of 5 x 10^3 cells/cm^2 in a 96-well culture dish (Costar). For experiments in which the density of the target cells were varied, the cells were seeded at one half the desired density. After overnight incubation, the cells were deprived of serum for 24 hours. Then the medium was removed and the test fraction was added (75 µl) along with 150 µl DME containing 5% (v/v) calf serum.

DNA synthesis was assayed 24 hours later with a pulse of [^{3}H]thymidine (1 µCi/culture, 1.9 Ci/mmole, Schwarz-Mann) for 3 hours at 37°. After the pulse, the radioactive medium was removed and the cells were washed three times with cold phosphate buffered saline (PBS) and once with 10% trichloroacetic acid. The cells were then solubilized with 0.2 ml of 1% sodium dodecyl sulfate (SDS) in 0.1 N NaOH. After incubation at 37° for 10 minutes, cell lysates were added to 2 ml of scintillation cocktail (1 g dimethyl-1,4-bis[2-5-phenyloxazoly]benzene; 8 g 2.5-diphenyl-oxazole; 1.33 ml Triton X-100; and 2.666 ml of toluene) for scintillation counting.

In reversibility studies, cultures were treated with fractions derived from FGR-s. After 24 hours, the medium was removed and replaced with an equal volume of fresh DME containing 5% (v/v) calf serum. DNA synthesis in these cultures was measured 12 hours and 24 hours later.

Assays of Cell Number and Cell Viability - Cultures were treated with fractions derived from FGR-s or a control fraction. At various times thereafter, the cells were washed three times with PBS and were removed from the growth surface by trypsin treatment. The cells were then centrifuged, resuspended in PBS, diluted with trypan blue, and counted in a corpuscle counting chamber (Hausser Scientific).

The viability of cells treated with FGR-s derived fractions was determined while the cells remained attached to the growth surface. After removal of growth medium, the cells were incubated with trypan blue (0.08% w/v in PBS) for ten minutes at room temperature. The staining solution was then removed and the viable cells were counted using an Olympus inverted microscope.

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing - Polyacrylamide gel electrophoresis in SDS was carried out according to the procedure of Laemmli (10), using 10% or 16% acrylamide running gels and 5% acrylamide stacking gels. For gels electrophoresed under reducing conditions, 2-mercaptoethanol (4%, v/v) was included in the sample buffer. The gels were fixed and stained with silver reagent (11). With radioactive samples, the gel was subjected to fluorography as described by Bonner and Laskey (12) using Kodak X-Omat R (XAR-5) film.

Isoelectric focusing was carried out in columns stabilized with a density gradient of sucrose, using Pharmalyte 3-10 (Pharmacia) (13). Fractions corresponding to FGR-s from a [^{35}S]methionine-labeled preparation was subjected to isoelectric focusing. The components separated by the pH gradient was subjected to scintillation counting to quantitate radioactivity and polyacrylamide gel electrophoresis in SDS and fluorography to define the polypeptide composition.

RESULTS

Isoelectric Focusing of FGR-s Polypeptides - In previous experiments, we had demonstrated that the growth inhibitory in conditioned medium of 3T3 cells can be fractionated by ammonium sulfate precipitation and gel filtration on Sephadex G-50 columns (4). The material eluting from the Sephadex G-50 column at a position corresponding to a molecular weight range of 10,000-15,000 was designated FGR-s. This fraction showed enrichment in specific biological activity (growth inhibition assay). The principal components of the FGR-s fraction were polypeptides of M_r 10,000 and 13,000.

When a [^{35}S]methionine-labeled preparation of FGR-s was further fractionated by isoelectric focusing in a sucrose gradient, at least three different components were observed (Fig. 1). Polyacrylamide gel electrophoresis in the presence of SDS showed that component A (Fig. 1) consisted mainly of a M_r 13,000 polypeptide (pI 10). Component B (Fig. 1) yielded a M_r 10,000 polypeptide (pI 7.5). Component C (Fig. 1) contained both polypeptides, as well as several higher molecular weight material, but at a level that was barely detectable in our fluorographic procedures.

Because the ampholines used to establish the pH gradient were toxic to the target 3T3 cells, we were unable to demonstrate growth inhibitor activity in these three components. Isoelectric focusing did not appear, therefore, to be a promising preparative procedure for the purification of the inhibitory polypeptide. Nonetheless, the high isoelectric point of FGR-s (13 K), which was implicated by monoclonal neutralization experiments to be at least partially responsible for

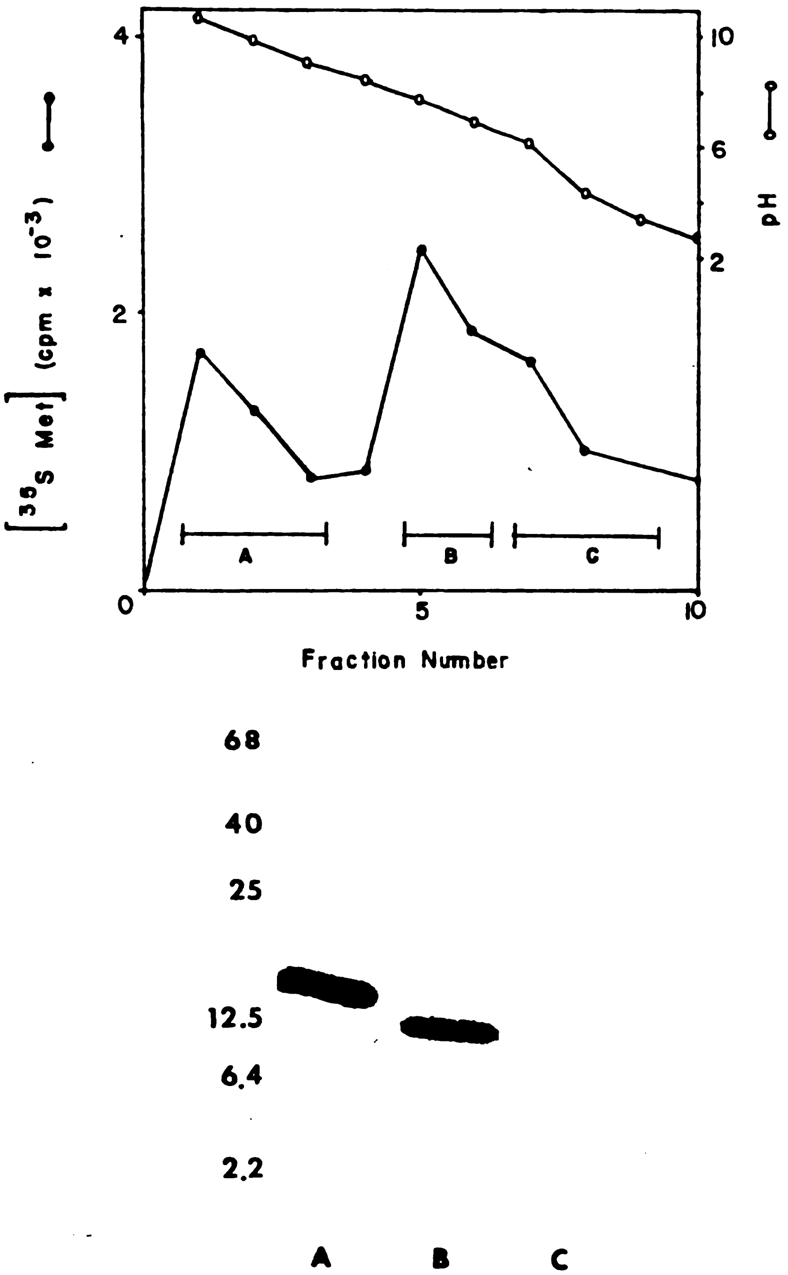


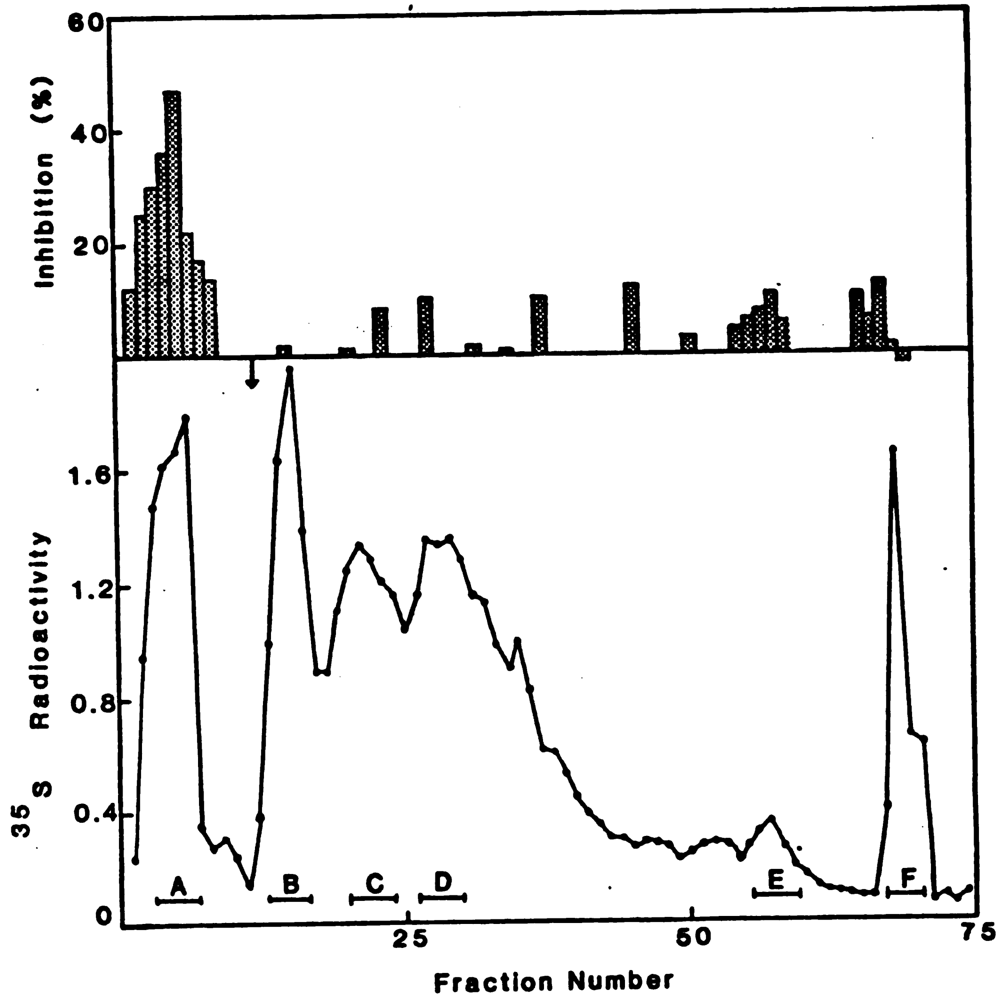
Figure 1: Isoelectric focusing profile of FGR-s in a sucrose gradient. The horizontal bars labeled A, B, and C denote the fractions that were pooled for polyacrylamide gel electrophoresis analysis, as shown on the lower panel. The labeled components are revealed after fluorography. The numbers on the left indicate the molecular weights (x 10^{-3}) and positions of migration of protein standards.

growth inhibitory activity (8), suggested ion-exchange chromatography as a preparative procedure.

Ion-exchange Chromatography of FGR-s - To carry out ion-exchange chromatography, two minor modifications of the original procedure (4) for the preparation of FGR-s were made. First, gel filtration on Sephadex G-50 columns was performed in 5 mM Tris, pH 8.0 instead of DME. This allowed the direct application of the effluent from the Sephadex column onto the ion-exchange column. Second, a wider range of fractions, centered approximately at FGR-s, was pooled and subjected to ion-exchange chromatography. The rationale for this was that we did not assay the individual fractions from the Sephadex 50 column for activity or for polypeptide content (SDS gels). Therefore, the precise position corresponding to the FGR-s fractions was not determined and was compensated for by including material in fractions adjacent to FGR-s. This allowed us to save material, which would have been used in the assays, and to save time, thereby minimizing losses of material due to adsorption to test tubes.

DEAE-cellulose chromatography of a [^{35}S]methionine-labeled preparation of FGR-s resulted in the separation of several components (Components A-F, Fig. 2b). When the fractions eluting from the ion-exchange column were assayed for growth inhibitory activity, only Component A (Fig. 2b) exhibited activity; the remainder of the components failed to show any appreciable activity (Fig. 2a). The sum of the growth inhibitory activity in Component A (Fig. 2b) accounted for 80% of the total activity applied to the column. There was a six-fold enrichment in terms of specific activity in this fractionation step.

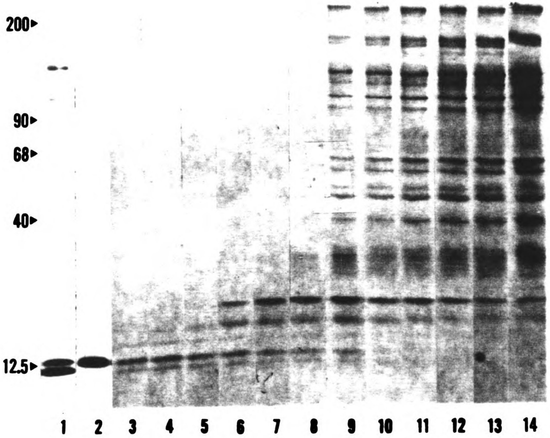
Figure 2: Ion-exchange chromatography of a [^{35}S]methionine-labeled FGR-s preparation (1.5 x 10^6 cpm) on a column (0.8 x 2 cm) of DEAE-cellulose equilibrated with 5 mM Tris, pH 8.0. At the point indicated by the arrow, a linear gradient (0-0.5 M NaCl, 100 ml total volume) was used to elute the material bound in the column. Fractions of 1.7 ml were collected. (a) Profile of the growth inhibitory activity assayed by the inhibition of [^{3}H]thymidine incorporation in target cells. The data on the ordinate axis are expressed as percent of inhibition relative to control cultures. The data represent the averages of triplicate determinations. (b) Profile of the protein content assayed by counting the radioactivity due to [^{35}S]methionine. The horizontal bars containing the letters A-F denote the fractions which were pooled for further analysis.


60
40
20
Inhibition (%)
1.6
1.2
0.8
0.4
0
35S Radioactivity
A
B
C
D
E
F
25
50
75
Fraction Number

Polyacrylamide gel electrophoresis and fluorography were carried out on the fractions derived from the DEAE-cellulose column (Fig. 3). Component A (Fig. 2b) yielded a single polypeptide, migrating at a position corresponding to a molecular weight of 13,000 (lane 2, Fig. 3). Identical results were obtained irrespective of whether the polyacrylamide gel electrophoresis was carried out under reducing (with β-mercaptoethanol) or non-reducing conditions (Fig. 4A). In addition to fluorography, we have also subjected the polyacrylamide gel to staining with the silver technique. Again, Component A yielded predominantly a single polypeptide with M_r 13,000 (Fig. 4B). Using known amounts of cytochrome c (M_r 12,500) as a standard for the silver staining technique, we estimate that 1 liter of conditioned medium derived from 3T3 cultures yielded approximately 1 μg of the 13,000 dalton polypeptide.

These results indicate that Component A (Fig. 2b) contains a single polypeptide chain (M_r 13,000) and exhibits growth inhibitory. They corroborate our previous conclusion, derived on the basis of neutralization of the growth inhibitory activity by a monoclonal antibody (8), that the M_r 13,000 polypeptide of FGR-s was at least partly responsible for the activity. We now can conclude that this M_r 13,000 polypeptide is active in growth inhibition independent of the presence of any other polypeptide. Moreover, the other polypeptides do not appear to contain growth inhibitory activity inasmuch as the remainder of the fractions from the DEAE-cellulose column (Components B-F, Fig. 2b) failed to show activity. The material in Component A (Fig. 2b) is hereafter designated FGR-s (13 K).

Figure 3: Polyacrylamide gel electrophoresis in SDS of a [^{35}S]methionine-labeled preparation of FGR-s and components derived from fractionation of FGR-s by ion-exchange chromatography (Figure 2). The acrylamide concentration of the running gel was 16%. The samples applied to the gel contained β-mercaptoethanol (4% v/v). Approximately 5,000 cpm were applied to each lane and the radioactive polypeptides were revealed by fluorography (2 days exposure). The arrows indicate positions of migration of molecular weight markers. Lanes 1: [^{35}S]methionine-labeled FGR-s; 2:fraction 6, 3:fraction 9, 4:fraction 15, 5:fraction 21, 6:fraction 27, 7:fraction 29, 8:fraction 33, 9:fraction 35, 10:fraction 38, 11:fraction 46, 12:fraction 52, 13:fraction 57, 14:fraction 68. All fractions refer to those fractions in Fig. 2b.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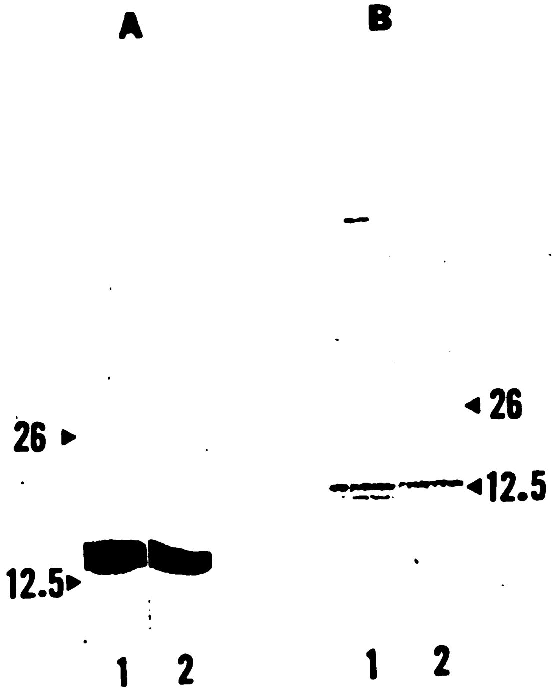

Figure 4: (A) Polyacrylamide gel electrophoresis in SDS of a [^{35}S]methionine-labeled preparation of FGR-s (13 K) under non-reducing (lane 1) and reducing conditions (4% v/v, 2-mercaptoethanol, lane 2). The acrylamide concentration of the running gel was 16%. Approximately 2,000 cpm were applied to each lane and the radioactive polypeptides were revealed by fluorography (14 days). The arrows indicate the positions of migration of molecular weight markers.

(B) Analysis of the polypeptide compositions of FGR-s (lane 1) and FGR-s (13 K) (lane 2) by the silver staining technique after SDS gel electrophoresis. The acrylamide concentration of the running gel was 16%. The samples applied to the gel contained 2-mercaptoethanol (4% v/v). The arrows indicate the positions of migration of molecular weight markers.

The Effects of FGR-s (13 K) on Target Cells: Dose-response, Viability, and Reversibility - The inhibitory effect of FGR-s (13 K) on [^{3}H]thymidine incorporation in target cells was dependent on the concentration of ligand added (Fig. 5). Over the concentration range of 0.1-10 ng/ml, there was a monotonic increase in inhibitory activity with increasing concentration of FGR-s (13 K) added to the cultures. The concentration of inhibitor required for 50% inhibition was 3 ng/ml (Fig. 5 inset).

Three series of experiments were performed to ascertain that the inhibition of [^{3}H]thymidine incorporation in target cells by FGR-s (13 K) was due to a true suppression of cell growth rather than to any cytotoxic effects of the inhibitory fractions. First, the viabilities of the cells, assayed by trypan blue exclusion tests, were identical for target cultures treated with FGR-s (13 K) and with a control fraction from the DEAE-cellulose column. Second, to demonstrate that the inhibitory effect of FGR-s on DNA synthesis was reversible, parallel cultures were treated with FGR-s (13 K) for 24 hours. The medium was then removed and replaced with an equal volume of fresh growth medium. At various times thereafter, DNA synthesis was assayed by the incorporation of [^{3}H]thymidine. The data showed that the inhibition was reversible within 20 hours after the removal of FGR-s (13 K). Taken together with the demonstration that the viability of target cells was not affected by FGR-s, these results suggest that the inhibitory activity of FGR-s cannot be ascribed to cytotoxicity.

Finally, the inhibitory effect of FGR-s (13 K) on cell proliferation is also reflected in assays of cell number after treatment with inhibitors. Target cultures treated with a control fraction from the

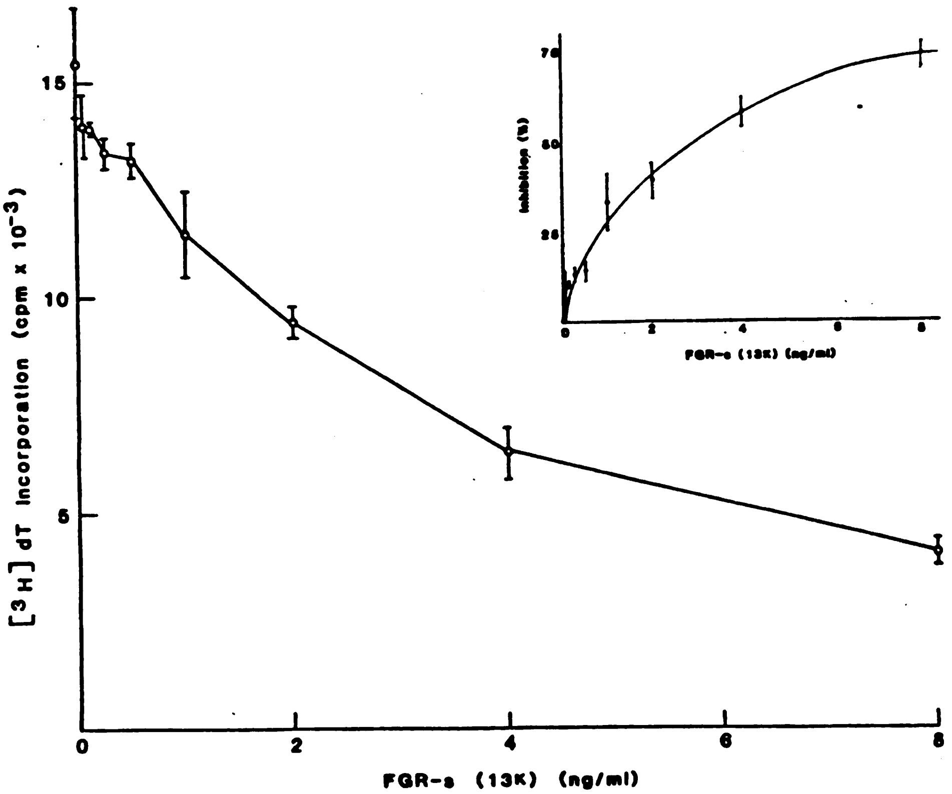

Figure 5: Dose-response curve of the growth inhibitory activity of FGR-s (13 K) on target 3T3 cells (2 x 10^4 cells/cm^2). The protein concentration of FGR-s (13 K) was determined by the silver staining technique on SDS gels. The target cells were treated with FGR-s (13 K) for 20 hours and were then assayed for the incorporation of [^{3}H]thymidine ([^{3}H]dT) as described in Experimental Procedures. The data represent the averages of triplication determinations (± standard error of the mean). The inset shows the same data plotted with percent inhibition on the ordinate axis.

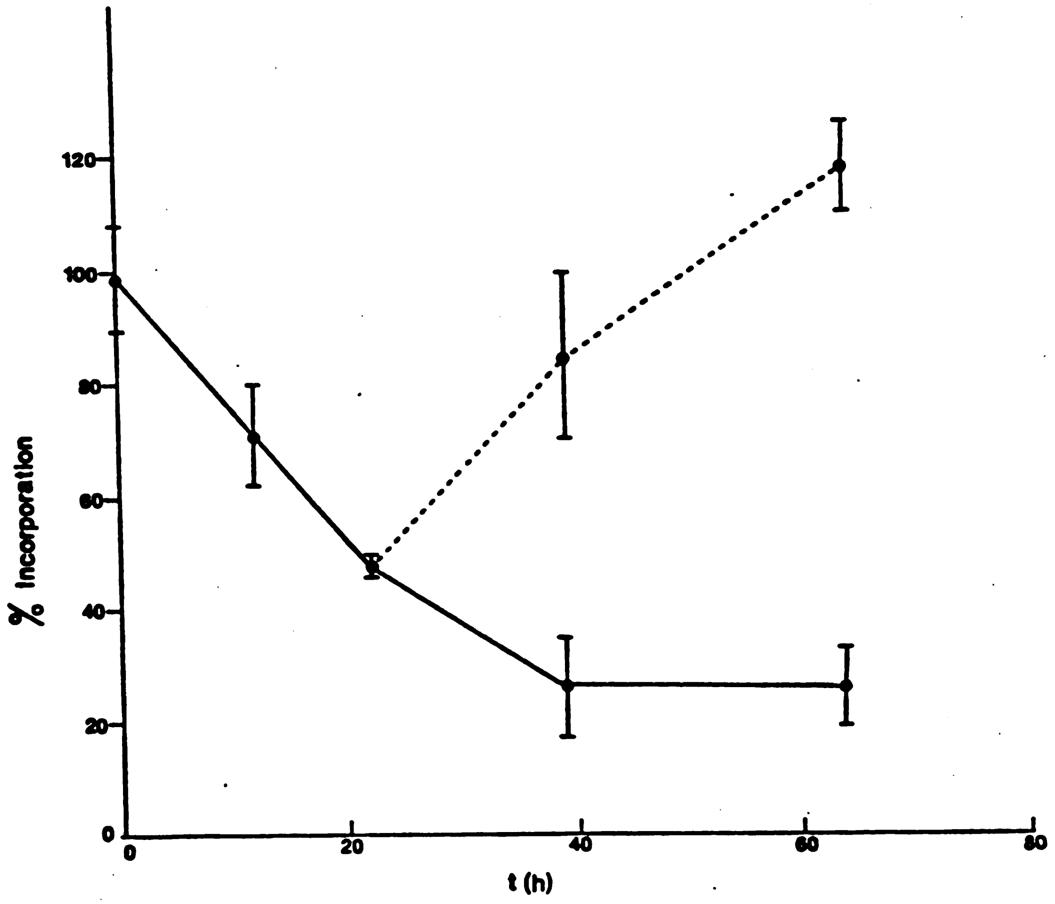

Figure 6:

The reversibility of the effect of FGR-s (13K) on 3T3 cells. Target cells (5 x 10^3 cells/cm^2) were treated with FGR-s (13K) (3 ng/ml). The kinetics of DNA synthesis, assayed by thymidine incorporation and expressed as per cent of control cultures, is shown by the solid line (———). The dotted line (----) indicates the level of DNA synthesis in cultures after the removal of FGR-s (13K), relative to control cultures which also received a medium change.

DEAE-cellulose column continued to proliferate, with a doubling time of about 20 hours. In contrast, cultures treated with FGR-s (13 K) failed to increase in cell number at the same rate. These data provide a confirmation of the growth inhibitory activity of FGR-s (13 K) using an assay that is independent of [^{3}H]thymidine incorporation.

The Effects of FGR-s (13 K) on Target Cells: Density and Serum Dependence - In this series of experiments, the effect of varying conditions of the assay during the period of exposure of target cells to FGR-s (13 K) was tested using a single preparation of the inhibitor. The effect of variation in the density of the target cells on the inhibition by FGR-s (13 K) is shown in Fig. 7. At target cell densities of 5 x 10^3 cells/cm^2 or above, the inhibitory activity of FGR-s (13 K) was consistently observed. In contrast, when the density of the target cells was below 2.5 x 10^3 cells/cm^2, there was a dramatic decrease in the potency of FGR-s (13 K) to inhibit 3T3 cells growth. This is consistent with our previous observation that a minimum target cell density may be required before the inhibitory effect of conditioned medium on cell proliferation can be observed (3).

The growth inhibitory activity of FGR-s (13 k) was dependent on the serum concentration used in the assay (Fig. 8). In the present experiment, FGR-s (13 K) (8 ng/ml) yielded 20% inhibition when the serum concentration was 5% or above. With decreasing concentration of serum, the same concentration of FGR-s (13 K) exhibited higher levels of growth inhibition (Fig. 8). In previous experiments, we showed that the binding of [^{35}S]methionine-labeled FGR-s was inhibited by serum (5). Therefore, the present result, showing higher levels of activity

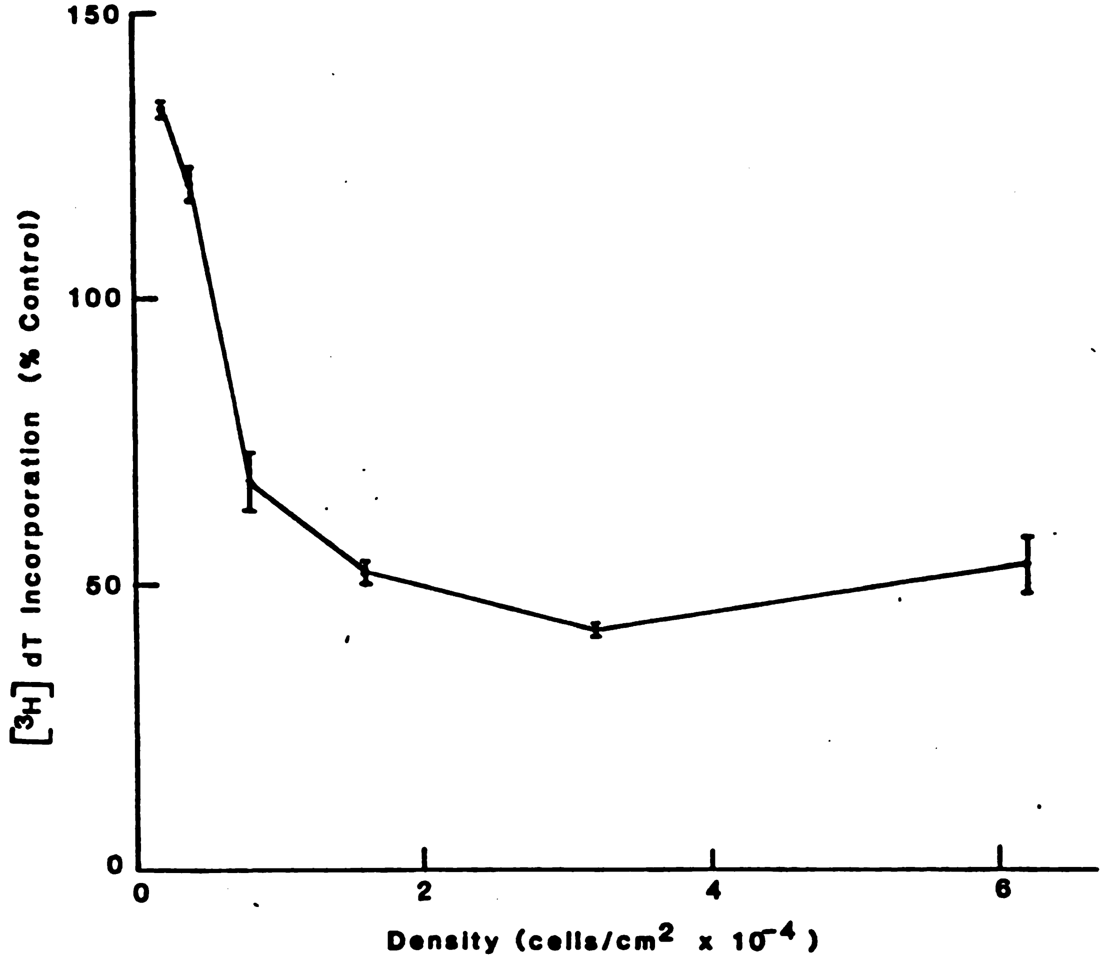

Figure 7:

The effect of varying the density of the target cells on the inhibitory activity of FGR-s (13 K). Target cells seeded at different densities were treated with FGR-s (13 K) for 24 hours, followed by a 3-hour pulse of [^{3}H]thymidine ([^{3}H]dT). Data points represent the averages of triplicate determinations (± standard error of the mean).

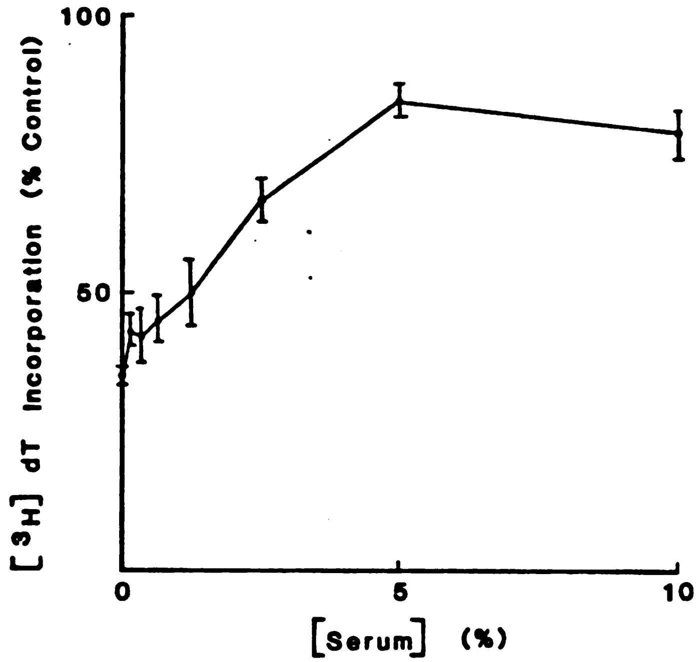

Fig. 8: The effect of serum concentration on the growth inhibitory activity of FGR-s (13K) assayed on target 3T3 cells (2 x 10^4 cells/cm^2). The target cells were treated with FGR-s (13K) for 24 hours in the presence of different concentrations of calf serum. The cells were pulsed with ^3H thymidine (^3H dT) for 3 hours. Data points represent the average of triplicate determinations (± standard error of the mean).

of FGR-s (13 K) as a function of decreasing concentration of serum, would be expected on the basis of higher levels of binding of the inhibitor.

DISCUSSION

The key conclusion derived from the present study is that FGR-s (13 K) exhibits growth inhibitory activity. In previous studies (8,9), we had generated a monoclonal antibody (2A4) that specifically bound FGR-s (13 K). This monoclonal antibody also neutralized the growth inhibitory activity of the FGR-s fraction. We inferred, therefore, that FGR-s (13 K) must be at least partly responsible for the biological activity. The present results corroborate the previous conclusion. Moreover, the purification of FGR-s (13 K) and the demonstration of its biological activity indicate that this polypeptide can exhibit growth inhibitory activity independent of the presence of any other polypeptide.

The main features of the inhibition of 3T3 cell proliferation by FGR-s (13 K) include: (a) The dose response curve indicates that 50% inhibition is obtained at a concentration of 3 ng/ml, corresponding to 2.3×10^{-10} M; (b) The effect of FGR-s (13 K) on 3T3 cells is reversible and cannot be ascribed to cytotoxicity; (c) The effect of FGR-s (13 K) on DNA synthesis was most prominent at high ($>5 \times 10^3$ cells/cm^2) target cell density, consistent with previous observations, made with conditioned medium (3), that a minimum target cell density may be required to observe the inhibitory effect; and (d) the inhibition by FGR-s (13 K) on 3T3 cells was most potent at low (<5%) serum concentrations, in agreement with previous results that the binding of radioactive FGR-s was decreased by increasing concentrations of serum (5).

Analysis of the molecular properties of FGR-s (13 K) indicates that it is active as a single polypeptide (M_r 13,000). This polypeptide remains as a monomer in SDS gel electrophoresis under both reducing and nonreducing conditions. Moreover, FGR-s (13 K) migrates on gel filtration columns at a position corresponding to a molecular weight of 13,000. This information is useful in a comparison of the properties of FGR-s (13 K) with corresponding properties of other negative regulators of cell growth (Table I).

Tucker et al. (14) have recently shown that Transforming Growth Factor-β (TGF-β) and a growth inhibitor isolated from conditioned medium of African green monkey kidney epithelial cells (BSC-1) shared many properties: (a) M_r 25,000 under non-reducing, non-denaturing conditions and M_r 12,500 under reducing SDS gels (15-17); (b) both TGF-β and the BSC-1 growth inhibitor stimulate colony growth of AKR-2B cells in soft agar; (c) both molecules inhibit the monolayer growth of a variety of cell types including BSC-1, AKR-2B, and mink lung (CLL64) cells; and (d) both appear to compete for the same receptors on the cell surface of responsive cells. Therefore, it appears that TGF-β and the BSC-1 growth inhibitor may be similar if not identical molecules (14). In corroboration of these results, Roberts et al. (18) have reported that TGF-β is a bifunctional molecule, acting either as a growth stimulatory factor or as a growth inhibitory molecule. The conditions that determine which of these two activities is expressed is not solely dependent on the cell type or the condition of anchorage dependent versus anchorage independent growth but is also modulated by the action of other growth factors (and their receptors) that may be present (18). TGF-β has been purified to homogeneity and therefore, it

Table I. Properties of Some Negative Regulators of Cell Growth

Source	Nomenclature	Target (indicator cells)	Native Molecular Weight	SDS Gels (reducing conditions)	References
3T3 conditioned medium	FGR-s (13 K)	3T3 cells	13,000	13,000	(this work)
various tissues, cells, and conditioned medium	transforming growth factor β (TGF-β)	NRK cells AKR-2B cells	25,000	12,500	16,17
human fibroblasts	interferon-β	HeLa	28,000-35,000	18,000	27,28
BSC-1 conditioned medium	BSC-1 growth inhibitor	CCL 64 mink lung	25,000	12,500	14,15
mouse embryo fibroblast conditioned medium	-	mouse embryo fibroblasts	10,000-15,000	14,000-11,000	7
rat liver	hepatocyte proliferation inhibitor	rat hepatocytes	26,000	26,000	23,24
bovine cerebral cortex cells	bovine glycopeptide inhibitor (BCSG)	mouse fibroblasts (3T3, LM), BHK-21	45,000	18,000-16,000	25
bovine mammary gland	-	Ehrlich ascites mammary cells	13,000	13,000	26

stands as a paradigm for negative regulators of cell growth and autocrine regulation.

Our present results on the purification of FGR-s (13 K) suggests that it may join TGF-β as another example of negative regulator that functions in an autocrine pathway (Table I). In comparing the properties of FGR-s (13 K) and TGF-β, it should be noted that the latter is active as a dimer (M_r 25,000) under non-reducing conditions. Upon reduction, the polypeptide molecular weight of TGF-β becomes 12,500 and the biological activity (stimulation of anchorage independent growth in soft agar cultures) is lost (16,17). In contrast, FGR-s (13 K) is active as a single polypeptide (M_r 13,000), as noted earlier.

Using procedures similar to our previous studies (3,4), Wells and Malucci (7) have shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose physico-chemical behavior and polypeptide composition closely parallel those of FGR-s. The molecular weights of the polypeptdies in their active fractions were 11,000 and 14,000 (Table I). In this connection, it should be noted that both soluble as well as plasma membrane associated growth inhibitory fractions, derived from 3T3 cells and with properties similar to FGR-s, have been reported from several laboratories (19-22). The molecular properties of these active fractions and their relationship to FGR-s (13 K) remain to be elucidated.

Three additional growth inhibitory activities have been characterized. (a) A hepatocyte proliferation inhibitor has been purified from rat liver; this inhibitor yielded a polypeptide (M_r 26,000) in SDS gels under reducing conditions and an isoelectric point of 4.56 (Table I). It reversibly inhibited the proliferation of non-malignant rat liver cells in culture but exerted no effect on the proliferation of hepatoma cells (23,24). (b) A glycopeptide fraction that inhibits protein synthesis and cell growth of normal but not transformed cells has been partially purified from bovine cerebral cortex cells (25). This preparation contained polypeptides of molecular weights 18,000 and 16,000 (Table I). (c) Finally, a growth inhibitor (M_r 13,000) for Ehrlich ascites mammary carcinoma cells has been partially purified from the bovine mammary gland (26). It is quite striking that the constituent polypeptide chains of several growth inhibitors have molecular weights of 13,000 and 26,000 (Table I). Perhaps a family of growth inhibitors may be defined when structural information becomes available for this list of negative regulators of cell growth.

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Chapter IV

GROWTH CONTROL IN CULTURED 3T3 FIBROBLASTS

VI. Effect of a Monoclonal Antibody on Cell Proliferation

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SUMMARY

In previous studies, we had generated a monoclonal antibody (Antibody 2A4) that bound a M_r 13,000 polypeptide of a partially purified growth inhibitory fraction called Fibroblast Growth Regulator - soluble form (FGR-s). In this study, we provide evidence for: (a) Antibody 2A4 binds FGR-s (13 K) and can deplete the growth inhibitory activity of that preparation; (b) Antibody 2A4 binds to the cell surface of live or unfixed 3T3 cells; (c) Antibody 2A4 enhances DNA synthesis in 3T3 cells cultured in the presence of serum growth factors. The results on the effects of Antibody 2A4 on target 3T3 cells in the absence of any exogenously added FGR-s molecules suggest that this monoclonal antibody can at least partially neutralize the activity of FGR-s (13 K) endogenous to the 3T3 culture and reverse the effect of density inhibition. This in turn implies that FGR-s (13 K) or a cross-reactive precursor molecule may play a role in the normal mechanism of density-dependent inhibition of growth in cultured 3T3 cells.

INTRODUCTION

A fibroblast growth regulator, which inhibits the growth and proliferation of mouse 3T3 cells, has been isolated from medium conditioned by exposure to density-inhibited 3T3 fibroblasts (1-3). The partially purified preparation of growth inhibitory activity, termed Fibroblast Growth Regulator - soluble form (FGR-s), contained two major polypeptides (M_rs, 10,000 and 13,000). Using FGR-s as the immunogen, we have carried out in vitro immunization of rat splenocytes and have generated hybridoma lines, each secreting an antibody directed against components of the FGR-s preparation.

One such monoclonal antibody, designated Antibody 2A4, specifically bound the M_r 13,000 polypeptide of FGR-s (4,5). Antibody 2A4 also neutralized the growth inhibitory effect of FGR-s. On the basis of these studies, we inferred that the M_r 13,000 polypeptide of FGR-s, hereafter termed FGR-s (13 K), is responsible for at least part of the growth inhibitory activity of the FGR-s fraction and may play a role in the normal mechanism of density dependent inhibition of growth.

If FGR-s (13 K) plays a physiologically significant role in density dependent growth control, one would expect that addition of Antibody 2A4 to cultures of 3T3 cells, in the absence of any exogenously-added FGR-s, should neutralize the activity of endogenous FGR-s (13 K) molecules in the culture and possibly reverse the effect of density inhibition. Our observations indeed lend support to this prediction. In the present communication, we report the effects of the interaction of Antibody 2A4 on target 3T3 cells in the absence of any exogenously-added FGR-s molecules.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of FGR-s (13 K) - Swiss 3T3 cells (American Type Culture Collection, CCL 92) were grown at 37° in Dulbecco modified Eagle's medium (DME, K.C. Biological) containing 10% calf serum (Microbiological Associates). The detailed protocol for the preparation of FGR-s (13 K) has been described (6). Briefly, confluent monolayers of 3T3 cells were washed twice with DME; fresh, serum-free DME was then added to the cultures, (10 ml/150 cm^2 of growth area). After overnight incubation, the medium was collected as serum-free conditioned medium. This medium was centrifuged at 1470 x g for 10 minutes and the supernatant was subjected to ammonium sulfate precipitation (80% of saturation at room temperature). The precipitate was redissolved in 5 mM Tris, pH 8.0 (2.5 ml of buffer per liter of conditioned medium) and fractionated by Sephadex G-50 chromatography (1.4 x 110 cm) in the same buffer. The material eluting at a position corresponding to polypeptides of molecular weight 10,000 to 15,000 was pooled as the FGR-s fraction. This material was further fractionated on a DEAE-cellulose column (0.8 x 2 cm) equilibrated with 5 mM Tris, pH 8.0. A gradient of 0 to 0.5 M NaCl in 100 ml of 5 mM Tris, pH 8.0 was used to develop the column. Fractions of 1.7 ml were collected.

Monoclonal Antibody 2A4 - The generation and characterization of hybridoma clone 2A4 have been previously described (4). Rat spleen cells were immunnized *in vitro* (7) with the FGR-s fraction. After 96 hours of culture, the rat lymphocytes were fused with the mouse myeloma cell line NS-1-Ag4/1 (NS-1 line, Salk Institute), using polyethylene glycol 1500 (8). Hybridoma cultures that produced antibodies reacting with FGR-s were detected by the binding of rat immunoglobulin in the culture supernatants to FGR-s adsorbed onto Immulon-2 plates (Dynatech) (4).

The supernatant from hybridoma clone 2A4 was fractionated on an affinity column of Affi-Gel 10 (BioRad) covalently derivatized (9) with rabbit antibodies directed against rat immunoglobulin (Sigma, St. Louis, MO). Material bound to the column was eluted with 0.1 M citrate buffer (pH 3.0). This material was a rat immunoglobulin designated as Antibody 2A4 (4).

Depletion of FGR-s (13 K) Over an Antibody 2A4 Column - Rabbit antibodies directed against rat immunoglobulin (40 mg) were coupled to Affi-Gel 10 (2 ml of beads) (9). Supernatant (50 ml) from the hybridoma clone 2A4 was passed through the column (0.4 x 2.5 cm) three times. The supernatant of the parent myeloma NS-1 cell line was similarly passed through an identical column for controls. Material bound non-specifically was removed by washing with 0.1 M phosphate buffer (pH 8.0). Finally, a preparation of FGR-s (13 K) (8 ng/ml, 2 ml) was percolated through either the Antibody 2A4 column or the NS-1 control column. The activities of the original FGR-s (13 K) preparation, as well as the pooled flow through fractions, representing material not bound by the respective affinity columns, were assayed for growth inhibitory activity.

Assays of DNA Synthesis and Cell Number - Target cells used to test the growth inhibitory activity were routinely seeded at an initial density of 5 x 10^3 cells/cm^2 in a 96-well culture dish (Costar). After overnight incubation, the cells were deprived of serum for 24 hours. Then the medium was removed and the test fraction was added (75 µl) along with 150 µl DME containing 5% (v/v) calf serum. DNA synthesis was assayed 24 hours later with a pulse of [^{3}H]thymidine (1 µCi/culture, 1.9 Ci/mmole, Schwarz-Mann) for 3 hours at 37°. After the pulse, the radioactive medium was removed and the cells were washed three times with cold phosphate buffered saline (PBS) and once with 10% trichloroacetic acid. The cells were then solubilized with 0.2 ml of 1% sodium dodecyl sulfate in 0.1 N NaOH. After incubation at 37° for 10 minutes, cell lysates were added to 2 ml of cocktail for scintillation counting (6).

Target cells used to test the effect of addition of Antibody 2A4 in the absence of any exogenously-derived FGR-s (13 K) were seeded at a density of 1 x 10^4 cells/cm^2. After addition of Antibody 2A4, the incorporation of [^{3}H]thymidine was assayed as described above.

For assays of increases in cell number after treatment with Antibody 2A4, target cells were seeded at a density of 1 x 10^3 cells/cm^2 in 6-well tissue culture dishes (Costar). At various times after the addition of Antibody 2A4 (0.5 mg/ml), the cells were washed three times with PBS and were removed from the growth surface by trypsin treatment. The cells were then centrifuged, resuspended in PBS, diluted with trypan blue, and counted in a corpuscle counting chamber (Hausser Scientific).

<u>Binding of Antibody 2A4 to 3T3 Cells</u> - Cells were seeded in 24-well dishes (Costar) at the density of 1 x 10^4 cells/cm^2 and grown at

37°C in DME for 48 hours to reach a confluent monolayer. Before binding with Antibody 2A4, cells were washed three times with DME at 4° and the culture dishes were left on ice-water in the cold room. Antibody 2A4 (15 μg/ml to 400 μg/ml) were added in 2 ml to the 3T3 cells incubated at 4°C for 40 minutes. The supernatants were removed and the cells were washed three times with cold DME. [^{125}I]Rabbit-anti-rat IgG antibody (10) was then added (100,000 cpm/well), incubated for another 40 minutes and washed with DME as above. The antibodies then were extracted with 0.2 M acetic acid (0.5 ml/well), and the acetic acid extracts were transferred to gamma counting tubes and counted by LKB Gamma Counter.

RESULTS

Depletion of Growth Inhibitory Activity from FGR-s (13 K) on an Affinity Column Containing Antibody 2A4 - In previous studies, we had demonstrated, at the analytical level, that [^{35}S]methionine-labeled FGR-s fractionated over an affinity column containing Antibody 2A4 resulted in the binding of a single polypeptide (M_r 13,000). We had also shown that the addition of Antibody 2A4 to growth inhibitory assays of FGR-s neutralized the biological activity. A control monoclonal antibody (Antibody 104) failed to yield the same effect (4,5). These results suggested that FGR-s (13 K) was responsible for the observed growth inhibitory activity.

In order to show that the M_r 13,000 polypeptide was directly responsible for the biological activity, an attempt was made to deplete, using an affinity column containing Antibody 2A4, the growth inhibitory activity of preparation of FGR-s (13 K). The affinity column was prepared by first coupling the immunoglobulin fraction of rabbit antibodies directed against rat immunoglobulin to Affi-Gel 10. This derivatized gel was used to prepare two different columns: (a) the supernatant of hybridoma clone 2A4 was passed over one column to bind Antibody 2A4 (Antibody 2A4 column); (b) the supernatant of parent myeloma NS-1 cell line was passed over the other column (NS-1 control column). Finally, a preparation of FGR-s (13 K) was percolated through either the Antibody 2A4 column or the NS-1 control column. The activities of the original FGR-s (13 K) preparation as well as the flow through fractions (representing unbound material) of the two respective affinity columns were compared in growth inhibition assays.

The results showed that the growth inhibitory activity of FGR-s was depleted in the material passed through the Antibody 2A4 column (Fig. 1). In contrast, the NS-1 control column had little effect on the growth inhibitory activity. These results, in conjunction with the previous demonstration that Antibody 2A4 affinity columns deplete FGR-s of the 13,000 dalton polypeptide, strongly suggest that FGR-s (13 K) is directly responsible for the observed growth inhibitory activity. The data also argues against any indirect mechanisms, such as Antibody 2A4 stimulating a growth factor receptor, in the observed neutralization of FGR-s effects in the growth inhibition assays (4). This conclusion is supported by the recent purification, to apparent homogeneity, of FGR-s (13 K) and the demonstration of its biological activity in the absence of any other polypeptide (6).

Effect of Antibody 2A4 on 3T3 Cultures in the Absence of Exogenously-added FGR-s - To test the possibility that Antibody 2A4 can neutralize the activity of FGR-s (13 K) molecules endogenous to 3T3 cell cultures, DNA synthesis in cells treated with various concentrations of Antibody 2A4 was assayed, 24 hours after addition of the monoclonal antibody, by the incorporation of [^{3}H]thymidine. The addition of Antibody 2A4 resulted in a higher level of DNA synthesis in a dose-dependent fashion (Table I). In contrast, the addition of Antibody 104, which binds to 3T3 cells but which is not reactive with FGR-s (4), failed to yield the same effect.

This effect of Antibody 2A4 on DNA synthesis in 3T3 cells is also reflected by monitoring the cell number of the cultures treated with the monoclonal antibody. Cultures treated with Antibody 2A4 had higher

Fig. 1:

Depletion of the growth inhibitory activity of FGR-s (13 K) by an Antibody 2A4 column. Affinity columns (0.4 x 2.5 cm) were prepared by coupling rabbit anti-rat immunoglobulin to Affi-Gel 10. The supernatant (50 ml) of hybridoma clone 2A4 or parent myeloma line NS-1 was passed over the affinity column. A preparation of FGR-s (13 K) (8 ng/ml, 2 ml) was percolated through these affinity columns. The growth inhibitory activities of the flow through fractions were compared to the original FGR-s (13 K) preparation (Ori) and control cultures treated with DME (Ctl). The data are expressed in terms of $[^3H]$thymidine ($[^3H]$dT) incorporation in target cells and represent the averages of triplicate determinations (± the standard error of the mean).

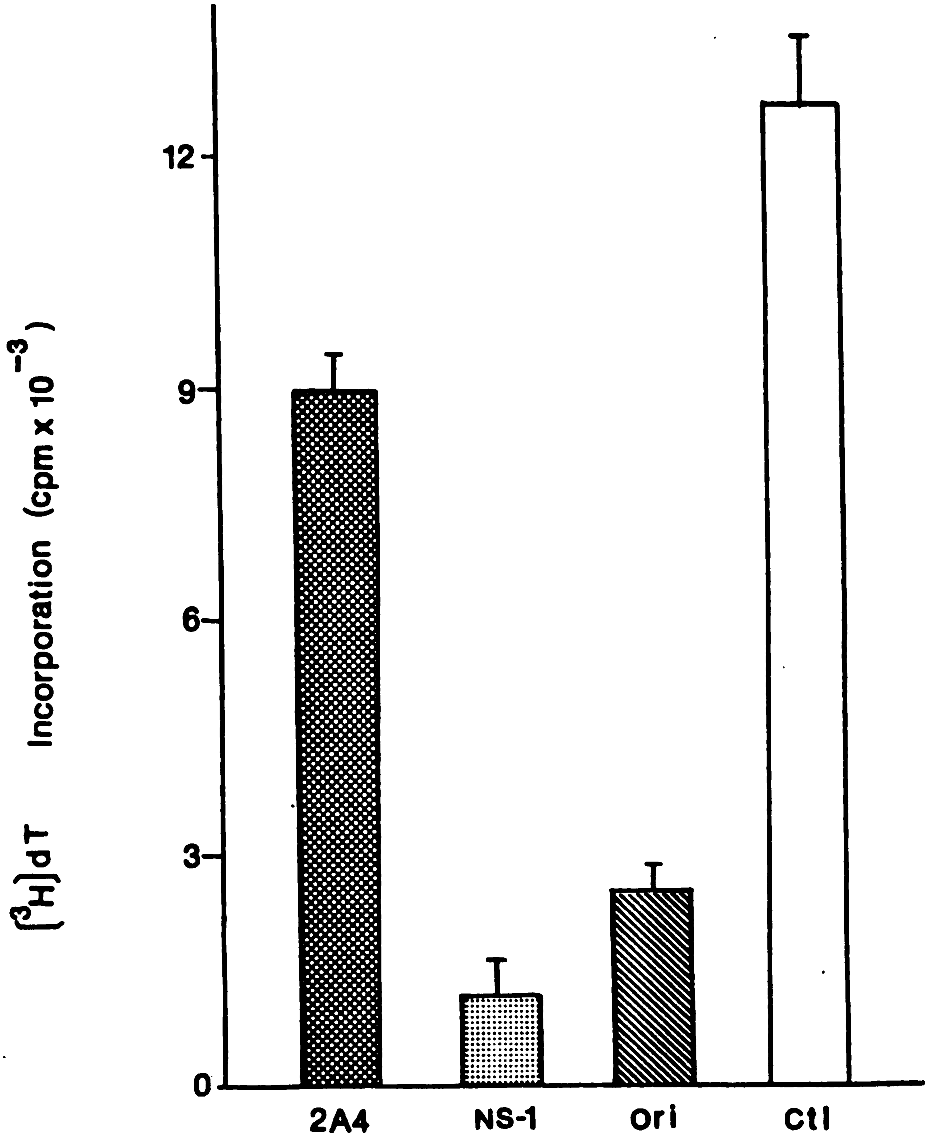
[3H]dT Incorporation (cpm x 10^-3)
0
3
6
9
12
2A4
NS-1
Ori
Ctl

Table I. Dose response of 3T3 cell DNA synthesis after Antibody 2A4 addition.

Antibody 2A4 concentration ng/ml	DNA synthesis (cpm)	stimulation index
0	5680	1.00
0.3	6770	1.19
1.6	6880	1.21
8.0	9660	1.70
40.0	9150	1.61
200.0	10060	1.77
1000.0	10840	1.91

The data are expressed in terms of ^{3}H-thymidine incorporation (cpm) in target cells and represent the averages of triplicate determinations. The target cells were seeded at a density of $\sim 10^4$ cells/cm^2.

saturation cell densities than control cultures (Table II). These results suggest that Antibody 2A4 can at least partially neutralize the activity of FGR-s (13 K) molecules endogenous to the 3T3 culture and reverse the effect of density inhibition. This in turn implies that FGR-s (13 K) plays a role in the normal mechanism of density-dependent inhibition of growth in cultured 3T3 cells.

Requirement for Serum Growth Factors in the Enhancement of 3T3 Proliferation by Antibody 2A4 - Dense cultures of 3T3 cells, arrested in the cell cycle by density inhibition and serum starvation, failed to respond to the addition of Antibody 2A4 (Table II). These cells can be activated, however, by the addition of serum. In the presence of increasing concentrations of serum, the addition Antibody 2A4 always enhanced the stimulation of DNA synthesis. These results indicate that the abrogation of the negative signal growth inhibition by FGR-s (13 K)) is not sufficient for activation of density-inhibited cells; positive stimulation by growth factors in the serum is required.

Interaction of Antibody 2A4 with 3T3 Cells - In our original screening assays to detect monoclonal antibodies directed against FGR-s, we had observed that the supernatant of hybridoma clone 2A4 showed positive reaction, relative to the supernatant of NS-1 control cultures, when assayed either against the specific FGR-s fraction or against whole 3T3 cells (4). In contrast, the supernatant of hybridoma clone 104 showed strong reaction when assayed on whole 3T3 cells but negligible reaction when assayed on FGR-s. We interpreted these results to indicate that

Table II. Serum dependence of the Antibody 2A4 effect.

Antibody treatment	Serum concentration (%)			
	0	0.5	2.0	10.0
None	1540	3630	5220	7450
2A4 (25 ng/ml)	1890	4740	7690	10090
Stimulation index	1.2	1.3	1.5	1.4

the product of clone 2A4 was a monoclonal antibody directed against FGR-s, which in turn is a constituent of whole 3T3 cells.

To test whether Antibody 2A4 binds to the cell surface of 3T3 fibroblasts, live or unfixed 3T3 cells were incubated with Antibody 2A4. After washing, the binding of the rat antibody to the cell surface was then detected by ^{125}I-labeled rabbit antibodies directed against rat immunoglobulin. The results showed that Antibody 2A4 bound directly to live 3T3 cells in a saturable fashion (Fig. 2) suggesting that there are a finite number of antigenic determinants exposed at the cell surface. Because Antibody 2A4 specifically recognizes FGR-s (13 K), we infer from these results that this polypeptide or a cross-reactive precursor is present on the plasma membrane.

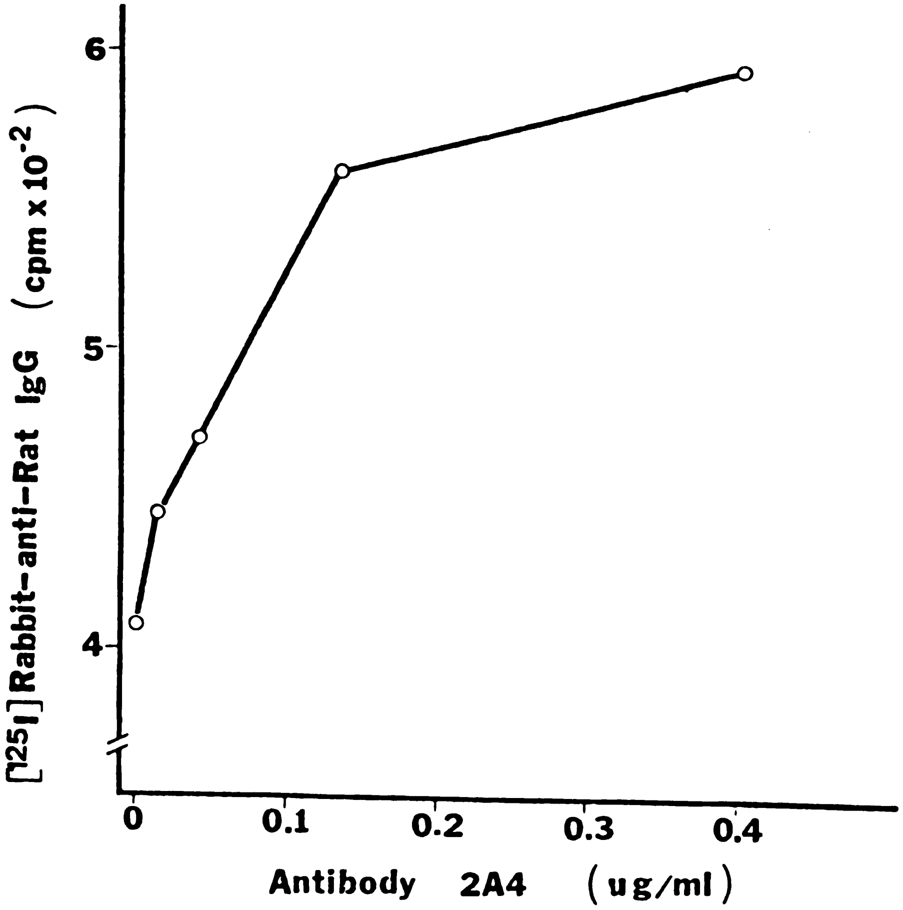

Fig. 2. Concentration dependence of the binding of Antibody 2A4 to confluent monolayers of 3T3 cells. The cells were incubated with Antibody 2A4 at 37° for 1h, washed and the amount of Antibody 2A4 bound to the cells was quantitated using ^{125}I-labeled rabbit anti-rat Ig (5 x 10^8 cpm/mg; 1 x10^5 cpm).

DISCUSSION

The experiments documented in the present study indicate that: (a) Antibody 2A4 binds to FGR-s (13 K) and can deplete the growth inhibitory activity of that preparation; (b) Antibody 2A4 binds to the cell surface of live or unfixed 3T3 cells; (c) Antibody 2A4 enhances DNA synthesis in 3T3 cells cultured in the presence of serum growth factors; and (d) FGR-s (13 K) or a cross-reactive molecule may play a key role in the normal mechanism of density-dependent inhibition of growth.

In previous studies, we had observed that the addition of Antibody 2A4 neutralized the activity of partially purified FGR-s fractions in growth inhibition assays (4,5). We could not be certain, however, that this was due to a direct effect of Antibody 2A4 on the FGR-s (13 K) molecule. For example, it was possible that the purified immunoglobulin fraction of Antibody 2A4 was contaminated by serum growth factors. The growth factors would stimulate DNA synthesis, thereby counter balancing the growth inhibitory effect of FGR-s. This possibility was considered unlikely on the basis of the observation that a control monoclonal immunoglobulin, Antibody 104, which bound to 3T3 cells but was not reactive with FGR-s polypeptides, failed to yield the same effects as Antibody 2A4. This conclusion was further supported by experiments that showed the same effects of Antibody 2A4 when the assays were carried out in the presence of freshly added calf serum (4,5).

It was also possible that Antibody 2A4 was interacting with receptors for growth factors on the cell surface and was mimicking the action of growth factors in stimulating DNA synthesis, as was demonstrated for certain monoclonal antibodies reactive with the receptor for Epidermal Growth Factor (11). This stimulation would again make it appear that Antibody 2A4 was abrogating the growth inhibitory effect of FGR-s (13 K). The present results shed light on this issue in two respects. First, the depletion of growth inhibitory activity of FGR-s (13 K) by an Antibody 2A4 column indicates that the FGR-s (13 K) molecule is directly involved in growth inhibition. Second, Antibody 2A4 enhanced DNA synthesis in quiescent 3T3 cells only in the presence of serum growth factors. In the absence of growth factors, Antibody 2A4 had no effect on DNA synthesis; thus, it does not appear that Antibody 2A4 can activate 3T3 cells via growth factor receptors.

The requirement for serum growth factors in the enhancement of DNA synthesis by Antibody 2A4 also illustrate another important feature of growth control in cultured 3T3 fibroblasts. In quiescent (serum-starved, density-inhibited) 3T3 cells, the abrogation of the negative signal (growth inhibition by FGR-s (13 K)) is not sufficient for activation of the cell cycle. Rather, positive stimulation by growth factors is required.

Antibody 2A4 binds directly to live or unfixed 3T3 cells. The binding appears to be saturable, suggesting that there are only a finite number of antigenic targets exposed at the cell surface. This is consistent with the finding that radioactively-labeled preparations of FGR-s can bind to or exchange with components of the cell surface (12). To date, we have not directly identified the antigenic target of Antibody 2A4 on the plasma membrane. Because Antibody 2A4 specifically recognizes FGR-s (13 K), however, we infer that the M_r 13,000

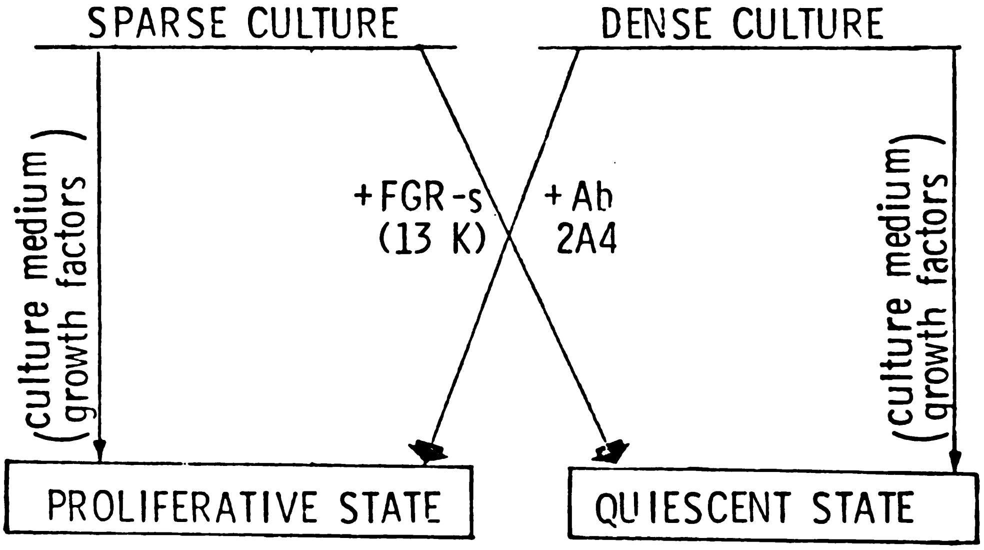

Fig. 3. Summary diagram showing the effect of FGR-s (13K) and Antibody 2A4 on spare and dense cultures of 3T3 cells.

polypeptide or a cross-reactive precursor is present on the plasma membrane.

Our overall results can be summarized by the diagram shown in Fig. 3. Under ordinary conditions, a sparse culture of 3T3 cells in medium containing serum growth factors will be a proliferating culture. In contrast, for a dense culture (4×10^4 cells/cm^2), these same 3T3 cells will cease to divide as they become quiescent. FGR-s (13 K) is a cellular protein fraction that will arrest sparse, proliferating 3T3 cells and convert them into a quiescent state. The M_r = 13,000 polypeptide in FGR-s is responsible for this action because the biological activity is neutralized by a monoclonal antibody directed against the polypeptide. This monoclonal antibody (Antibody 2A4) will also stimulate DNA synthesis in dense, quiescent cultures of 3T3 cells. This suggests that the target of 2A4 (FGR-s (13 K) or a cellular precursor) may be functioning in the normal mechanism of density-dependent growth control in 3T3 cells.

The fact that FGR-s (13 K) can be isolated from soluble conditioned medium lends support to the notion that growth regulation at high cell densities may be partly mediated by soluble inhibitory factors (13-16). We had previously shown that radioactively-labeled FGR-s preparations bound specifically to the target 3T3 cells (12). Antibody 2A4, which recognizes FGR-s (13 K), also binds to the surface of 3T3 cells in a saturable fashion. These results suggest that FGR-s (13 K) or a higher molecular weight precursor is present on the plasma membrane. This in turn raises the possibility that the endogenous growth inhibitor may also act via cell-cell contact mediated mechanism of growth regulation which is supported by the early observations of 3T3 cell growth as well as by the wound healing experiments of Dulbecco and Stoker (17,18).

Indeed, Glaser and co-workers have shown that the growth of 3T3 cells can be reversibly inhibited by a surface membrane fraction from the same cells and that the inhibitory components can be solubilized by the nonionic detergent octylglucoside (19). Alternatively, Peterson et al. (20) have obtained similar growth inhibitory activities from 3T3 cell membranes by solubilzing the membrane with Triton X-100 and reconstituting the solubilized components into liposomes. Natraj and Datta have also shown that an inhibitor of DNA synthesis can be extracted from 3T3 cells by treatment with 0.2 M urea in phosphate-buffered saline (21). It was suggested that these surface-membrane molecules may be the same molecules that are responsible for contact-dependent growth regulation. It would be of obvious interest to establish the relationship between FGR-s (13 K) and the plasma membrane associated growth inhibitory activities. It is possible that the same molecule can exert its effects both anchored on the cell surface and released into the medium.

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CLOSING STATEMENT

The isolation and characterization of FGR-s (13K) and of TGF-β make it possible to begin studies of negative regulation of cell growth at the molecular level. Three key issue should be focused in the future studies of negative growth regulators.

The first issue is structure. The amino acid sequence of TGF-β has been determined and molecular cloning is well on its way in Sporn's laboratory at the National Institute of Health. The isolation of FGR-s (13K) has recently been brought to a stage where attempts to screen a gt11 expression library derived from cDNA of mouse fibroblasts and to determine the partial amino acid sequence for the synthesis of oligonucleotide probes to screen a cDNA library have only just begun. Clearly, structural information would facilitate our understanding at several different levels. (a) The relationship between the growth inhibitors and known protein products, protein kinases, or oncogenes, as had been strikingly demonstrated for growth factors. (b) The relationship between FGR-s, TGF-β , and other growth inhibitors. It is quite striking that the constituent polypeptide chains of several growth inhibitors have molecular weight of 13,000 or 26,000. A family of growth inhibitors, similar to that of growth stimulatory factors, might be defined. Structural relationships between growth inhibitors could also be tested using immunological cross-reactivity and receptor competition assays. (c) A search for better sources of identified growth inhibitors and for new inhibitors. The generation of antibody and cDNA probes would allow for such a search at the transcript or gene product level.

The second issue is the identification and characterization of receptors for growth inhibitors. For both FGR-s and TGF-β, binding to cell surface receptors has been demonstrated. The molecular identity and possible enzymatic activities of the receptors remain, however, to be determined. A survey of the distribution of receptors (number and/or affinity differences) amongst normal versus neoplastic cells, cells of different tissues, might provide clues concerning the specificity of action of the inhibitors on target cells.

The third issue is effect of transformation. The ability of tumor cells to produce and to respond to their own growth factors provided a central concept linking oncogene and growth factor research. The "autocrine hypothesis" proposed by Todaro and Sporn states that oncogenes confer growth factor autonomy on cells not only by coding directly for peptide growth factors or their receptors (enzyme activity) but also by amplifying the mitogenic signals generated by a growth factor at its receptor. The "autocrine hypothesis" may now be extended to include the concept that malignant transformation may be the result not only of excessive production, expression and action of positive autocrine growth factors, but also of the failure to express or respond to specific negative regulators of cell growth. This general concept has been championed for a number of years but the recent developments on TGF-β and FGR-s have put this notion on a firm experimental basis. The loss of negative growth control may result from several different levels of bio-chemical lesions, including (a) mutation or loss of the structural gene for growth inhibitor itself; (b) a loss of transcriptional or translational controls for expression of growth inhibitor; (c) an increased rate of degradation or clearance of the inhibitor; (d) a defect in the cell surface receptors; or (e) lesions in the post-receptor signalling pathway. All of these levels need to be tested using purified inhibitor, antibody, and molecular cloned probes.