Canine Tracheal Epithelial Cells Express the Type 1 Insulin-like Growth Factor Receptor and Proliferate in Response to Insulin-like Growth Factor I

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Disaggregated airway epithelial cells replicate in serum-free media containing supraphysiologic concentrations of insulin. To examine the hypothesis that the type 1 insulin-like growth factor (IGF) receptor mediates the mitogenic action of insulin on these cells, we studied the mitogenic effects of IGF-I and insulin, and the expression of type 1 IGF receptors in primary cultures of adult canine tracheal epithelial cells. Isolated tracheal epithelial cells were grown in varying concentrations of IGF-I or insulin in Ham's F12 medium supplemented with transferrin, cholera toxin, and endothelial cell growth supplement. Both IGF-I and insulin increased DNA synthesis (measured as [^{3}H]thymidine incorporation into DNA) and cell number in a concentration-dependent fashion, but IGF-I was at least 20 to 60 times more potent than insulin in its mitogenic effects. No additive or synergistic effect was observed with the simultaneous addition of IGF-I and insulin in maximally effective doses. A monoclonal antibody directed against the type 1 IGF receptor (αIR3) blocked the mitogenic activity of both IGF-I and insulin. Affinity labeling of type 1 IGF receptors by covalent cross-linking with disuccinimidyl suberate demonstrated the tracheal epithelial cell IGF-I binding moiety to have a relative molecular weight of 130,000 D. Binding of [^{125}I]IGF-I to this protein was inhibited by low concentrations of IGF-I, relative to insulin, and by αIR3. An 11-kb transcript characteristic of mRNA for the type 1 IGF receptor was recognized in poly(A$^+$) RNA derived from cultured canine tracheal epithelial cells. These data demonstrate, by functional, biochemical, and molecular methods, that cultured canine tracheal epithelial cells express type 1 IGF receptors which mediate their mitogenic response to IGF-I or supraphysiologic concentrations of insulin. These results also raise the possibility that IGF-I may have an important role in growth and differentiation of large airways epithelium.

The factors controlling airway epithelial growth and differentiation during development and repopulation after injury are not defined. Peptide growth factors have been implicated in these processes (1). Evidence of a role for insulin-like growth factor I (IGF-I) comes from the findings that it is synthesized in the lung (2) and that type 1 IGF cell membrane receptors have been demonstrated in lung tissue (3).

Supraphysiologic concentrations of insulin have been used

to support the growth of a wide variety of epithelial cells, including tracheal epithelium *in vitro* (4). In cells other than pulmonary epithelia, high concentrations of insulin can interact with the type 1 insulin-like growth factor receptor to stimulate mitosis (5–7). We postulated, therefore, that in airway epithelia, insulin's effect on cell proliferation is also mediated through the type 1 IGF receptor and that IGF-I is a more potent mitogen for epithelial cells than insulin by virtue of preferential binding to its high-affinity receptor. To examine these questions, we determined the mitogenic effects of IGF-I using cultured canine tracheal epithelial cells in defined, serum-free media, and examined the expression of the type 1 IGF receptor by tracheal epithelial cells.

Materials and Methods

Materials

Cell culture–grade insulin, transferrin (TF), endothelial cell growth supplement (ECGS), and cholera toxin (CT) were obtained from Collaborative Research (Lexington, MA). Ham's F12 and Eagle's minimum essential media (MEM)

were from GIBCO (Grand Island, NY). Fetal bovine serum
(FBS) was obtained from Hyclone Laboratories (Logan,
UT). Protease XIV, trypsin, deoxyribonuclease (DNAse),
monoclonal anti-human myoglobin antibody, and bovine se-
rum albumin (BSA), fraction V (lot No. 88f-0071, IGF-I-
free) were purchased from Sigma Chemical Co. (St. Louis,
MO). Disuccinimidyl suberate was from Pierce (Rockford,
IL). Recombinant [Thr^{59}]IGF-I was from Amgen Biologi-
cals (Thousand Oaks, CA). Guanidine thiocyanate was pur-
chased from Fluka (Ronkonkoma, NY), and cesium chloride
from Gallard-Schlesinger (Carle Place, NY). [Methyl-
^{3}H]thymidine (sp act, 91 Ci/mmol), Na^{125}I, and [α-^{32}P]UTP
were obtained from Amersham (Arlington Heights, IL).
Monoclonal antibody against the type 1 IGF receptor (αIR3)
was a gift from J. J. Van Wyk and S. Jacobs (8). [^{125}I]-la-
beled IGF-I was prepared from recombinant IGF-I, purified
by immunoaffinity chromatography, and labeled by the chlo-
ramine T method (9). A human cDNA for the type 1 IGF
receptor was obtained from American Type Culture Collec-
tion (No. 59294; Rockville, MD).

Isolation of Cells

Mongrel dogs were killed by an i.v. injection of pentobarbital
(125 mg/kg), tracheas removed, and tracheal epithelial cells
isolated as described by Wu and Smith (10). Briefly, connec-
tive tissue was removed and the tracheal segments were
rinsed in MEM and treated with 0.1% protease XIV and
0.01% DNAse in MEM for 16 h at 4° C. FBS (10%) was then
added, the mixture was agitated vigorously, and the disag-
gregated cells were filtered through Nitex mesh. The cells
were centrifuged at 500 × g for 10 min, the pellet washed
with MEM and 10% FBS, resuspended, and centrifuged
again. The final pellet was resuspended in 5% FBS and
Ham's F12 medium (F12) supplemented with penicillin (50
U/ml), streptomycin (50 U/ml), and gentamicin (50 μg/ml).
Cell viability was determined by exclusion of trypan blue,
and was consistently > 90%. Tracheal epithelial cells iso-
lated by this technique are virtually free of fibroblast con-
tamination as judged by morphology and antikeratin immu-
nostaining (10). Serum-free conditions used after cell
attachment to plastic substrate do not support fibroblast sur-
vival (11).

Culture Conditions

The disaggregated cells suspended in F12 with 5% FBS were
plated in 24-well (16-mm-diam well) tissue culture plates
(Costar, Cambridge, MA) at a density of 2 × 10^5 cells/cm^2.
After 24 h, the unattached cells were removed and the culture
medium was replenished with F12 supplemented with TF (5
μg/ml), CT (0.01 μg/ml), ECGS (7.5 μg/ml), and various con-
centrations of insulin or IGF-I. Every other day, cells were
washed with F12 and fresh medium was added. All experi-
ments were performed using subconfluent monolayer cul-
tures, as judged by phase-contrast microscopy. Cell cultures
were maintained at 37° C in 95% humidity and 5% CO$_2$.

Growth Properties of Canine Tracheal Epithelial Cells

Cells were grown on plastic culture plates in F12 with TF,
CT, and ECGS (control medium), or control medium sup-
plemented with insulin (860 nM or 10 μg/ml) or IGF-I (13

nM or 100 ng/ml). Each day for 5 d, cells were removed
from four wells by trypsinization (0.1% trypsin and 1 mM
EDTA) and cell number was determined in quadruplicate by
electronic cell counting (Model Z$_{BI}$; Coulter, Hialeah, FL).
The cell doubling time was calculated by the method of Chen
and Chen (12).

To determine the concentration relationship of the growth
response to IGF-I and insulin, freshly isolated tracheal epi-
thelial cells were plated in plastic multiwell plates and al-
lowed to attach over a 24-h period. The media was then
changed to provide F12 with TF, CT, and ECGS with multi-
ple concentrations of insulin or IGF-I. On the fifth day in cul-
ture, 1 μCi of [methyl-^{3}H]thymidine was added to the media.
Twenty-four hours later, the cells were removed by trypsiniza-
tion, and cell number and [methyl-^{3}H]thymidine incorpora-
tion into DNA were determined. Cellular DNA was precipi-
tated in cold perchloric acid–methanol, the precipitant
recovered by centrifugation, and radioactivity of the pellet
measured (13).

The effect of the simultaneous addition of insulin and
IGF-I to canine tracheal epithelial cells was determined by
growing cells in control medium and 1.7 μM insulin with
multiple concentrations of IGF-I. [Methyl-^{3}H]thymidine
(1 μCi per well) was added 16 h before harvesting on the
sixth day. Cell number per well and tritiated thymidine in-
corporation into DNA were determined for each culture con-
dition. The same procedure was followed for tracheal epithe-
lial cells grown in control medium and 6.5 nM IGF-I with
multiple concentrations of insulin.

Inhibition of Type 1 IGF Receptor-ligand Binding

Canine tracheal epithelial cells were isolated as described
above, but after protease/DNAse treatment, 2.5% BSA was
added to the solution in place of 10% FBS to prevent possi-
ble interactions of serum components with cell surface re-
ceptors and antibody. The disaggregated cells were suspended
and plated in F12 with TF, CT, and ECGS (control medium).
After cell attachment, the cells were washed, and monoclo-
nal antibody directed against the IGF receptor (8) was added
to one half of the wells at a concentration of 550 nM (100
μg/ml). Four hours later, additional media, control medium,
or control with either IGF-I (13 nM) or insulin (170 nM) was
added to paired groups of wells. After a 4-d incubation
period during which the culture media was not changed, the
cells were removed and cell number was determined by elec-
tronic cell counting. Exactly the same procedure was fol-
lowed using a monoclonal antibody directed against human
myoglobin (at the same molar concentration) to serve as an
isotypic control antibody.

Affinity Cross-linking Analysis

Previously described methods (14, 15) were used with the fol-
lowing modifications. Canine tracheal epithelial cells plated
at a density of 2 × 10^5 cells/cm^2 on 60-mm plastic dishes
were incubated for 18 h in F12 with 5% FBS and then 3 d
with F12 plus control medium with 1.7 μM insulin, as de-
scribed above. Media was then aspirated and the cell mono-
layer washed three times with 3 ml Hepes binding buffer (20
mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM
MgSO$_4$, 10 mM NaHCO$_3$, 1.3 mM CaCl$_2$, and 1.2 mM

KH$_2$PO$_4$) with 1% BSA (fraction V, IGF-I free; Sigma). Cells were then incubated with [^{125}I]-labeled IGF-I (6 × 10^5 cpm/dish) in Hepes binding buffer (with 1% BSA) with or without various concentrations of unlabeled IGF-I, insulin, or monoclonal antibody at 8° C for 20 h. After incubation, the media was aspirated and cells were washed with 3 ml ice-cold Hepes buffer without BSA. Disuccinimidyl suberate (0.1 mM) in Hepes binding buffer without BSA was added for 15 min at room temperature. The reaction was quenched by adding 9 ml of 10 mM Tris-HCl, pH 7.4, with 1 mM EDTA for 15 min. The solution was aspirated and the cells were solubilized in 200 µl of 2% SDS, 0.05 M Tris-HCl, and 0.1 M DTT. The cell lysates were removed by scraping with a rubber policeman and transferred to microfuge tubes. The samples were concentrated by evaporation (Savant, Farmingdale, NY) for 3 h to one-half their original volume and then boiled for 5 min. Sample proteins and [^{14}C]-labeled molecular weight standards (myosin, M_r = 200,000; phosphorylase b, M_r = 100,000; BSA, M_r = 69,000; ovalbumin, M_r = 46,000; and carbonic anhydrase, M_r = 30,000) were separated using 10% SDS-PAGE (16). The gel was fixed in 10% acetic acid and 30% methanol, washed, dried, and exposed to Kodak AR5 film in the presence of Dupont Cronex Lightning Plus intensifying screens at −70° C.

Northern Hybridization Analysis

IGF-I receptor cDNA corresponding to a 706-bp segment of the α subunit was cloned into the EcoRl site of pBS$^+$ (Stratagene, La Jolla, CA) and a [^{32}P]-labeled antisense riboprobe was *in vitro* transcribed using T7 RNA polymerase and [α-^{32}P]UTP (17). Subconfluent primary cultures of canine tracheal epithelial cells, grown for 6 d after plating as described above in plastic T-150 flasks in control medium with 1.7 µM insulin, were solubilized with 4 M guanidine thiocyanate directly in culture flasks. Total cellular RNA was extracted by centrifugation through a 5.7 M CsCl cushion (18) and poly(A$^+$)RNA isolated using oligo (dT) cellulose (19). Samples of poly(A$^+$)RNA were denatured in glyoxal and dimethyl sulfoxide at 50° C and size-fractioned by electrophoresis in 1% agarose gels. After capillary transfer to nylon membranes (Gene Screen; New England Nuclear, Boston, MA) (20), baking at 80° C, and UV cross-linking, the membrane was prehybridized with buffer (6× SSC, 50 mM Tris-HCl, pH 7.0, 10× Denhardt's reagent, and 50% formamide). Hybridization to a [^{32}P]-labeled riboprobe was performed for 16 h at 65° C. After standard posthybridization washes in 50 mM NaCl, 20 mM NaHPO$_4$, 1 mM EDTA, and 1% SDS at 55° C, the membrane was air-dried and exposed to Kodak AR5 film with intensifying screens at −70° C for 16 h (21).

Statistics

Studies of tracheal epithelial cell growth and inhibition of type 1 receptor binding were performed in quadruplicate for each experiment. Analysis of variance with Tukey's method of multiple comparison was used to compare data from experiments with more than two samples. A P value < 0.05 was used for all tests.

Results

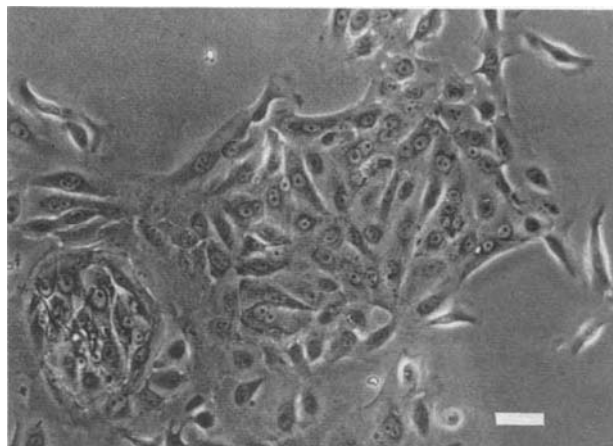The canine tracheal epithelial cells studied are polygonal and

Figure 1. Phase-contrast microscopy of canine tracheal epithelial cells on plastic substrate. Bar = 50 µm.

have the morphologic characteristics of epithelial cells. Figure 1 is a phase-contrast photomicrograph of these cells under subconfluent conditions. Canine tracheal epithelial cells cultured in Ham's F12 medium supplemented with TF, CT, and ECGS did not proliferate, whereas with the addition of either insulin (860 nM) or IGF-I (13 nM), their cell doubling times were 109 ± 31 and 106 ± 41 h (mean ± SD; n = 4 or 5), respectively (Figure 2). Both IGF-I and insulin stimulated a concentration-dependent proliferation of tracheal epithelial cells (Figure 3). Each peptide produced an
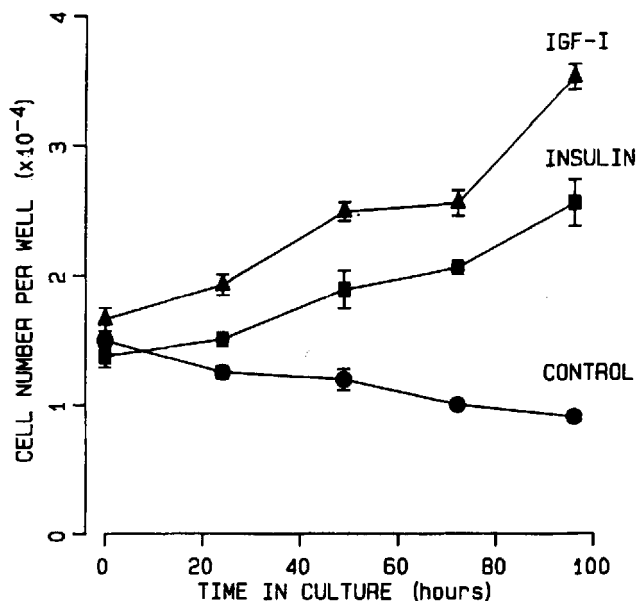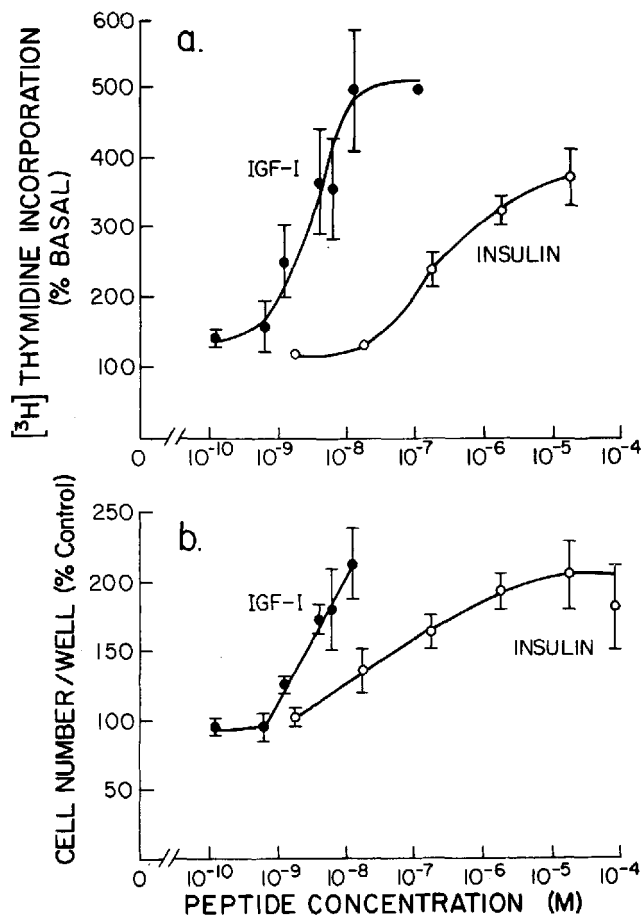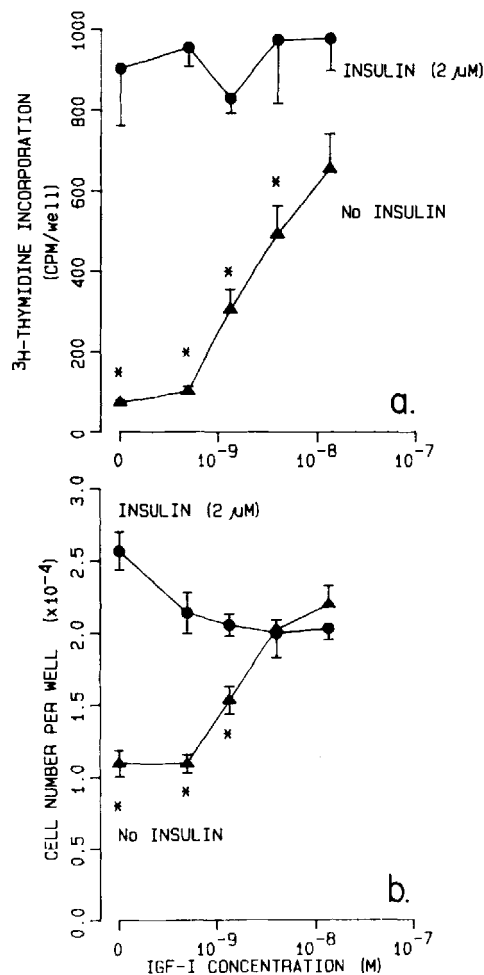

Figure 2. Growth of canine tracheal epithelial cells on plastic substrate. Cells (2 × 10^5 cells/cm^2) were plated in 24-well culture plates and maintained in Ham's F12 with TF, CT, and ECGS (control medium) (*circles*), control with 860 nM insulin (*squares*), or control with 13 nM IGF-I (*triangles*). Cell number per well was determined in quadruplicate. Mean ± SEM are shown from a representative experiment.
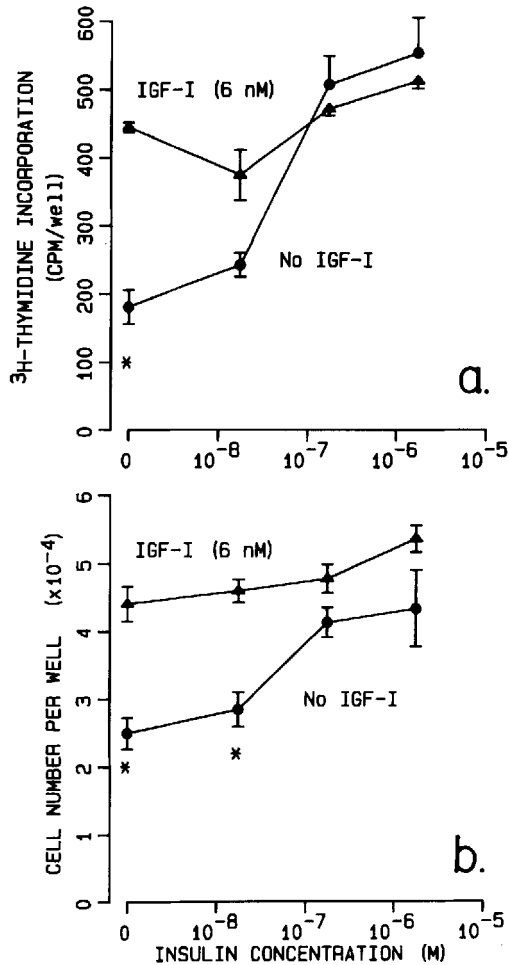
Figure 3. IGF-I and insulin stimulation of DNA synthesis and cell proliferation. Canine tracheal epithelial cells were initially plated in Ham's F12 with 5% FBS for 24 h, and then maintained for 6 d in control medium and multiple concentrations of either IGF-I (*closed circles*) or insulin (*open circles*) with media changes every other day. Data points represent mean ± SEM of experiments using tracheal epithelial cells from nine animals (each point represents two to seven experiments, each performed in quadruplicate). (a.) [Methyl-³H]thymidine incorporation into DNA. After 5 d of growth, 1 μCi/ml [methyl-³H]thymidine was added to each well and incubated another 16 h. Methanol-perchloric acid–precipitated material was collected and scintillation counting performed. (b.) Tracheal·epithelial cells were treated as described above. After a 6-d incubation, cells were harvested and counted using a Coulter counter.

increase in DNA synthesis and cell number, but IGF-I was more potent than insulin. The concentration of IGF-I required to affect proliferation was 20-fold lower than that of insulin when half-maximal stimulation was calculated (3 nM for IGF-I and 60 nM for insulin). Similar differences (60-fold) were observed when thymidine incorporation was used to calculate half-maximal stimulatory effects (3 nM for IGF-I and 180 nM for insulin). No additional increase in thymidine incorporation into DNA was seen with 100 nM IGF-I in a single experiment. When the minimum dose required for maximal proliferation was used as a measure of potency (10 nM IGF-I versus 10 μM insulin), IGF-I was approximately 1,000 times more potent than insulin.
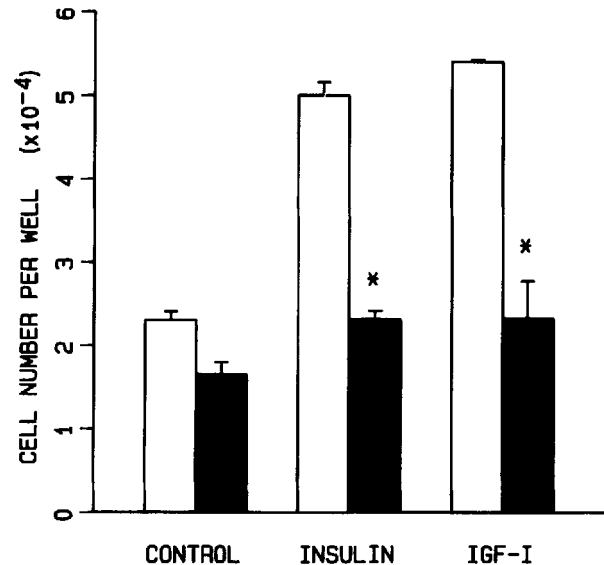

Figure 4. Additive effects of IGF-I and insulin on cell proliferation and DNA synthesis in canine tracheal epithelial cells. Each point is expressed as the mean ± SEM from quadruplicate wells in a representative experiment from the same animal. Asterisks indicate significant differences ($P < 0.05$) between cells incubated in the presence and absence of insulin at the same IGF-I concentrations. (a.) Cells initially plated in F12 with 5% FBS for 24 h were maintained in Ham's F12 plus TF, CT, ECGS, and either IGF-I and insulin (1.7 μM) (*circles*) or IGF-I alone (*triangles*). After 5 d of growth in defined medium with media changes every other day, 1 μCi/ml [methyl-³H]thymidine was added to each well for a 16-h incubation. Methanol-perchloric acid–precipitated material was collected and scintillation counting performed. (b.) Cells were treated as described above. At the conclusion of the growth period, cell number was determined using a Coulter counter. No differences were seen among cells incubated with 1.7 μM insulin and multiple IGF-I concentrations.

To determine if the effects of IGF-I's and insulin were additive or synergistic, increasing concentrations of IGF-I were added to a concentration of insulin that stimulated mitosis. As illustrated in Figure 4, no further increase in cell proliferation or DNA synthesis was observed when 13 nM IGF-I was combined with 1.7 μM insulin in the culture medium. Similar results were obtained when increasing doses of insulin were added to a mitogenic concentration of IGF-I (Figure 5). These results suggest that both IGF-I and insulin act through a common mechanism.

Figure 5. Additive effects of insulin and IGF-I on cell proliferation and DNA synthesis in canine tracheal epithelial cells. Each point is expressed as the mean ± SEM from quadruplicate wells in a representative experiment from the same animal. Asterisks indicate significant differences ($P < 0.05$) between cells incubated in the presence and absence of IGF-I at the same insulin concentrations. (a.) Tracheal epithelial cells were initially plated in F12 with 5% FBS for 24 h and then maintained in F12 plus TF, CT, ECGS, and either insulin and IGF-I (6.5 nM) (*triangles*) or insulin alone (*circles*). After 5 d of growth in defined medium, 1 μCi/ml [methyl-^{3}H]-thymidine was added to each well for a 16-h incubation. Methanol-perchloric acid–precipitated material was collected and scintillation counting performed. (b.) Cells were treated as described above. At the end of the growth period, cell number was determined using a Coulter counter. No differences were seen among cells incubated with 6.5 nM IGF-I and multiple insulin concentrations.

Figure 6. Inhibition of cell proliferation by monoclonal antibody to the type 1 IGF receptor. Cells were plated in 24-well plastic plates in serum-free medium (F12 with TF, CT, and ECGS). After a 1-d incubation for cell attachment, monoclonal antibody to the type 1 IGF receptor (αIR3, 550 nM) or control antibody (anti-human myoglobin, 550 nM) was added to one-half of the wells. Four hours later, additional media containing F12 with TF, CT, and ECGS (control medium) or control with either IGF-I (13 nM) or insulin (170 nM) was added to paired groups of wells. After 4 additional d of incubation, cell number was determined.

The effect of each antibody was studied in cells from two animals; therefore, the proliferative response of each to insulin and IGF-I was tested to determine if comparisons between the two cell preparations was valid. Cell number per well (expressed × 10^{-4}; mean ± SD) measured at the conclusion of the growth period for each condition was: control medium, 1.18 ± 0.09 and 2.21 ± 0.83 ($P = 0.13$); insulin (170 nM), 4.14 ± 0.43 and 4.69 ± 0.68 ($P = 0.96$); IGF-I (13 nM), 3.95 ± 0.26 and 4.50 ± 0.33 ($P = 0.88$) for the isotypic antibody and αIR3, respectively. Because there were no statistical differences between the two cell preparations, they are shown together in this graph. In the respective experiments, αIR3 inhibited both insulin-stimulated and IGF-I–stimulated proliferation compared to cells incubated without antibody ($P < 0.001$), while the isotypic antibody had no inhibitory effect on these parameters.

Results are expressed as the mean ± SEM. The shaded bars represent cells grown in the presence of αIR3 and open bars cells incubated with control antibody. Asterisks denote significant differences ($P < 0.001$) in cell number in the presence of antibody.

To investigate whether IGF-I's and insulin's mitogenic actions are mediated by binding to the type 1 IGF receptor, the stimulatory effects of each were studied in the presence of an antibody directed against this receptor. This antibody, αIR3, has been shown to inhibit binding of both IGF-I and insulin to the type 1 receptor (5, 6, 8). To control for any possible nonspecific effects of antibody addition to canine tracheal epithelial cells, an isotypic antibody (to myoglobin) was studied in an identical fashion. Figure 6 compares cell growth in the presence of the isotypic control antibody and

anti-IGF receptor antibody and either insulin or IGF-I, as well as in their absence. The proliferative response to IGF-I and insulin was inhibited by αIR3 in each case despite the addition of sufficient concentrations of either IGF-I (13 nM) or insulin (170 nM) to stimulate cell proliferation. The cellular proliferative response was not inhibited in the presence of the same concentration of isotypic control antibody.
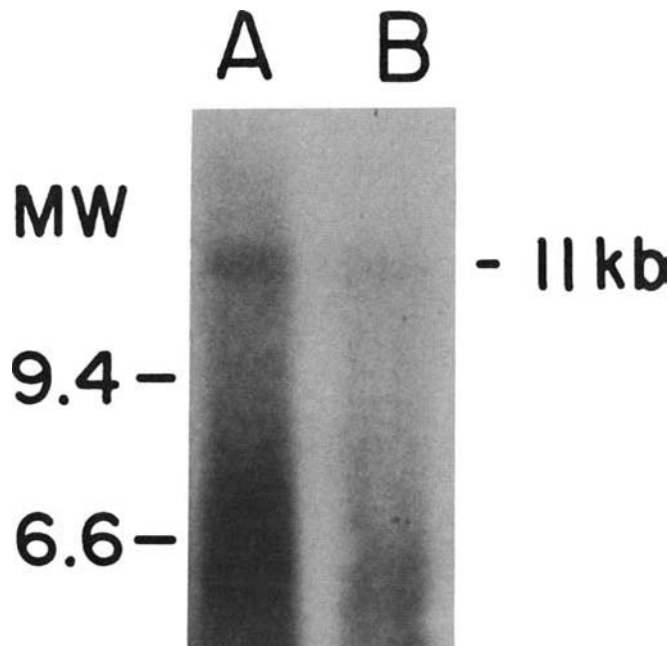
To confirm that IGF-I and insulin were interacting with the type 1 IGF receptor, affinity labeling was performed by cross-linking [^{125}I]IGF-I with its binding moiety in the presence of various concentrations of IGF-I or insulin and monoclonal antibodies. Under reducing conditions, [^{125}I]IGF-I was
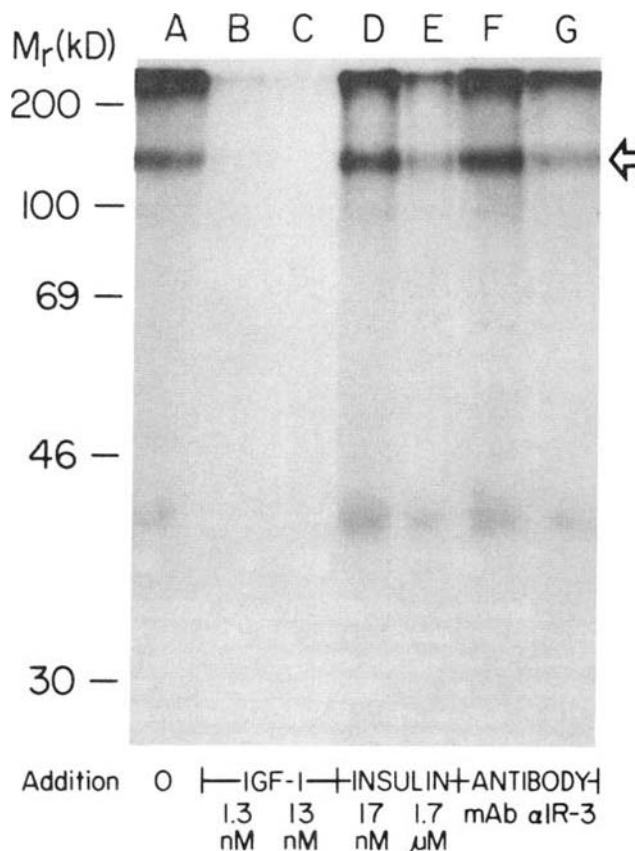
Figure 7. Affinity cross-linking of IGF-I to canine tracheal epithelial cells. Tracheal epithelial cells were incubated for 18 h at 8° C with [^{125}I]IGF-I in the absence (*lane A*) or the presence of unlabeled IGF-I, 1.3 nM (*lane B*) or 13 nM (*lane C*); unlabeled insulin, 17 nM (*lane D*) or 1.7 μM (*lane E*); or 550 nM monoclonal antibody, control anti-myoglobin (*lane F*) or anti-type 1 IGF receptor/αIR3 (*lane G*). The arrow points to the [^{125}I]IGF-I affinity-labeled α subunit of the type 1 IGF receptor. The labeled moiety at about 40,000 D is an affinity-labeled IGF binding protein. Affinity cross-linking was performed with 0.1 mM disuccinimidyl suberate as described in MATERIALS AND METHODS. Shown are autoradiographs of the radioligand-receptor complexes analyzed by PAGE under reducing conditions. The migration of molecular weight standards is indicated on the left.

Figure 8. Northern hybridization of mRNA from canine tracheal epithelial cells with human type 1 IGF receptor riboprobe. An autoradiograph of poly(A$^+$)RNA from primary cultures of canine tracheal epithelial cells hybridized with a [^{32}P]-labeled human type 1 IGF receptor riboprobe is shown. Lanes A and B contain 10 μg and 5 μg, respectively, of poly(A$^+$)RNA obtained from different animals. Cells had been isolated, plated, and grown for 5 d in F12 with TF, CT, ECGS, and 1.7 μM insulin as described in MATERIALS AND METHODS. The hybridizing band of 11 kb is indicated along with molecular weight standards.

bound to a protein of about 130,000 D (Figure 7), a relative size that corresponds to the binding subunit of both the type 1 IGF receptor and the insulin receptor (22). Complete inhibition of labeled peptide binding occurred with as little as 1.3 nM IGF-I, whereas 1,700 nM insulin was required to decrease the binding of [^{125}I]IGF-I. This result confirms that the affinity-labeled moiety is the type 1 IGF receptor, rather than the insulin receptor, and is consistent with the known differences in receptor affinity for each peptide (23). Furthermore, the type 1 IGF receptor monoclonal antibody (αIR3) competed with labeled peptide, whereas control antibody had no effect. This result demonstrates that the inhibitory effects of αIR3 are mediated by its interaction with the type 1 IGF receptor.

To unequivocally confirm that canine tracheal epithelial cells express type 1 IGF receptors, Northern analysis of

poly(A$^+$)RNA from primary cultures of canine tracheal epithelial cells was performed using a human IGF-I receptor riboprobe. This riboprobe recognized an mRNA of about 11 kb (Figure 8), a transcript of the same size reported by others in mRNA derived from mouse, rat (lung), and human tissue (24, 25). This result demonstrates that epithelial cells from canine trachea express a type 1 IGF receptor mRNA similar to those previously described, indicating significant sequence homology among species.

Discussion

We have demonstrated that canine tracheal epithelial cells in primary culture express type 1 IGF receptors and that these receptors mediate a mitogenic response. Expression of the type 1 IGF receptor was demonstrated by functional, biochemical, and molecular criteria. Namely, the response of tracheal epithelial cells to IGF-I and insulin is consistent with the relative affinities of each peptide to the type 1 IGF receptor (23); the canine tracheal epithelial cell IGF-I binding moiety has a relative molecular size of the binding subunit of the type 1 IGF receptor (about 140,000 D) (22), and binding of labeled IGF-I to this protein is inhibited by low concentrations of IGF-I, relative to those of insulin, and by a monoclonal antibody that is specific to this receptor; and, finally, an 11-kb transcript, characteristic of the mRNA for the type 1 IGF receptor in other species (24, 25), is recog-

nized in poly(A$^+$)RNA purified from canine tracheal epithelial cells.

We have also demonstrated that the proliferative response of canine tracheal epithelial cells to IGF-I and insulin is mediated by the type 1 IGF receptor. IGF-I stimulates DNA synthesis and cell proliferation in tracheal epithelial cells in concentrations 20- to 60-fold lower than those required for a similar response to insulin, a result consistent with the relative affinities of these peptides to the type 1 IGF receptor (23). Furthermore, a monoclonal antibody specific for the type 1 IGF receptor inhibits the response of canine tracheal epithelial cells to both IGF-I and insulin. These results also clarify why supraphysiologic concentrations of insulin stimulate cell proliferation in primary epithelial cell culture systems.

The selection of transferrin, endothelial cell growth supplement, and cholera toxin as supplements to the culture medium was determined, in part, by previous studies that suggested that these factors were necessary for optimal canine tracheal epithelial cell growth (26). Despite the mitogenic action of these supplements in other culture systems (4), a combination of these factors was not sufficient to allow tracheal cell proliferation without the addition of IGF-I or high concentrations of insulin.

Our results are consistent with an *in vivo* role for IGF-I in the stimulation of tracheal epithelial cell proliferation, because the nanomolar concentrations of IGF-I necessary to stimulate tracheal epithelial cell proliferation are likely to be within the physiologic range. Although lung tissue IGF-I concentrations are not known in humans or dogs, human serum concentrations are about 25 nM (27), while canine serum concentrations average 10 nM (28).

The tracheal epithelial cell types responsive to mitogenic stimulation are not known. Cells in this primary culture system are not morphologically differentiated but can be induced into ciliated and secretory cell types by manipulating substrate and culture media supplements (11, 26). Because basal cells and nonciliated cells have the shortest doubling times *in vivo* (29), they appear to be the best candidates for IGF-I responsiveness. Definitive demonstration of type 1 IGF receptors on specific epithelial cell types will require *in situ* hybridization and immunocytochemistry correlated with rigorous morphologic identification.

The lung is known to elaborate bioactive and immunoreactive IGF-I (30, 31) and to express type 1 IGF receptors (3). In the human fetus, a paracrine role for IGF-I has been postulated based on the findings that lung mesenchymal elements (septa, perivascular fibroblasts, and possibly interstitial cells) possess mRNA for IGF-I (32), while IGF-I identified by immunocytochemistry is localized to airway epithelium (33). Furthermore, Nakanishi and colleagues (34) recently showed that small-cell lung carcinoma cell lines produce an IGF-I precursor, proliferate in response to IGF-I, and demonstrate growth inhibition in the presence of anti-IGF receptor monoclonal antibody, consistent with an autocrine mode of action. Our study adds to the mounting evidence of a role for IGFs in the growth of lung epithelial cells by demonstrating that primary cultures of canine tracheal epithelial cells proliferate in response to IGF-I, an effect mediated by the type 1 IGF receptor. These results prompt us to speculate that IGF-I may be an important mitogen for the tracheal epithelium in early life and after injury.

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