Induction of Transforming Growth Factor-β1 (TGF-β₁) Receptor Expression and TGF-β₁ Protein Production in Retinoic Acid-Treated HL-60 Cells: Possible TGF-β₁-Mediated Autocrine Inhibition

By Lydia A. Falk, Fabrizio De Benedetti, Nancy Lohrey, Maria C. Birchenall-Roberts, Larry W. Ellingsworth, Connie R. Faltynek, and Francis W. Ruscetti

Treatment of HL-60 cells, a human promyelocytic leukemia cell line, with the vitamin A derivative retinoic acid (RA) for 7 days resulted in a dose-dependent decrease in proliferation and increase in granulocytic differentiation. The role of transforming growth factor-β₁ (TGF-β₁), a protein with pleiotropic effects on the proliferation and differentiation of various cell types, was examined during RA-induced differentiation of HL-60 cells. Although TGF-β₁ alone had little effect on proliferation or differentiation of HL-60 cells, addition of TGF-β₁ to HL-60 cells treated with a suboptimum concentration of RA (1.0 nmol/L) resulted in a marked decrease in proliferation with no effect on granulocytic differentiation. Studies of the mechanism of RA-induced TGF-β₁ sensitivity showed that although untreated HL-60 cells expressed low levels of TGF-β₁, binding proteins on the cell surface, the levels were increased in a dose-dependent manner after RA treatment. Maximum induction was achieved after treatment with 10 nmol/L RA and consisted predominantly of the 65-kD TGF-β₁ receptor type. Moreover, RA treatment also resulted in a dose-dependent increase in both TGF-β₁, steady-state mRNA expression and production of active TGF-β₁ with maximum induction at 10 nmol/L RA. RA treatment of HL-60 cells had no effect on TGF-β₁ and TGF-β₁ mRNA expression. These data suggest that the effects of RA may be mediated by a TGF-β₁-mediated autocrine antiproliferative loop during differentiation of HL-60 cells. This is a US government work. There are no restrictions on its use.

RETINOIC ACID (RA) treatment of patients with acute promyelocytic leukemia resulted in remission with morphologic granulocytic maturation in >50% of the patients treated. HL-60, a human promyelocytic cell line, can differentiate into three distinct myeloid cell lineages: granulocyte, monocyte-macrophage, and eosinophil, depending on the differentiation stimuli. Specifically, dimethylsulfoxide (DMSO) or RA treatment of HL-60 cells favors increased terminal granulocytic differentiation, making the HL-60 cell line a good model for studying the physiologic requirements for granulocytic leukemic cell differentiation.

The effects of a number of cytokines on induction of HL-60 cell differentiation have been evaluated. Specifically, tumor necrosis factor-α (TNF-α), interferons, and RA in various combinations exhibit synergistic interactions favoring the monocytic differentiation of HL-60 cells. Recent studies indicate that transforming growth factor-β₁ (TGF-β₁) can also synergize with TNF-α to induce monocytic differentiation of HL-60 cells. TNF-α treatment of HL-60 cells resulted in rapid induction of TGF-β receptors, providing a possible mechanism for the synergistic activity of TGF-β₁ and TNF-α in monocytic differentiation of HL-60 cells.

The mechanism(s) by which RA inhibits HL-60 cell proliferation and supports granulocytic differentiation is unknown. Recent studies showed, however, that several normal and leukemic primary hematopoietic cells and cell lines are sensitive to the antiproliferative effects of TGF-β. To determine whether TGF-β₁ was involved in RA-induced granulocytic differentiation of HL-60 cells, we examined RA-treated HL-60 cells for TGF-β₁ receptor expression, TGF-β₁ mRNA expression, and active TGF-β₁ protein production. RA treatment of HL-60 cells resulted in (a) a dose-dependent increase in TGF-β₁ receptor expression, (b) an increase in steady-state levels of TGF-β₁ mRNA with no effect on TGF-β₁ or TGF-β₁ mRNA expression, and (c) an increase in protein with TGF-β₁-like activity. Because addition of TGF-β₁ to RA-pretreated cells inhibited HL-60 cell proliferation but had no effect on the differentiation state of the cells, our data provide evidence that RA may stimulate a TGF-β₁-mediated antiproliferative loop during differentiation of HL-60 cells.

MATERIALS AND METHODS

Cell culturing techniques. The HL-60 and HL-60/MR1 cell lines were provided by Dr T. Breitman (National Institutes of Health, Bethesda, MD) and were grown as previously described. Cells were grown in RPMI (Whittaker M. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT), 100 U/mL penicillin/streptomycin (GIBCO, Grand Island, NY), and 2.0 mmol/L L-glutamine (GIBCO). On day 0, 2 x 10⁶ cells/mL were treated with various concentrations of RA diluted in DMSO (Sigma Chemical, St Louis, MO) so that the final concentration of DMSO was never greater than 0.01%. This concentration was shown to have no effect on proliferation, differentiation, or TGF-β₁ receptor expression of the cells (data not shown). HL-60 cells were subcultured again on day 1248 B/ood, Vol77, No6 (March 15, 1991: pp 1248-1255
4 with RA-containing medium. After 7 days of RA treatment, HL-60 cells were harvested and assayed for TGF-β sensitivity, receptor expression, mRNA expression, protein production, and evidence of differentiation.

**Proliferation assays.** RA-treated HL-60 cells (2 × 10^5) were replated in 96-well culture dishes and incubated with various concentrations of bovine TGF-β, (Collagen Corporation, Palo Alto, CA) for 4 days. Cultures were pulsed with 1 μCi per well of [3H]-thymidine (specific activity 40 to 60 Ci/mmol/L; Amersham, Arlington Heights, IL) during the last 12 hours of culture. [3H]-Thymidine incorporation was determined by liquid scintillation counting.

**Morphologic and cytochemical studies.** HL-60 cells were seeded at 2 × 10^6 cells per well in 24-well culture dishes. Cultures were treated with RA for 7 days. Cultures were then incubated with or without TGF-β, for 3 more days. At the end of the culture period, cells were cytostirufuged (Shandon Southern Instruments, Sewickley, PA) and analyzed for morphologic changes with Jenner's Giemsa staining and for monocyte differentiation with a commercially available α-naphthyl acetate esterase kit (ANAE, Sigma). The determination of nitroblue tetrazolium reduction (NBT) was evaluated by incubation for 30 minutes at 37°C of 1 × 10^5 cells/0.1 mL medium, and equal volumes of 0.2% NBT (Sigma) and 200 ng/mL phorbol myristate acetate (PMA; Sigma). The percentage of cells containing intracellular blue/black formazan deposits was determined.

**Chemical cross-linking analysis of TGF-β receptors.** Cross-linking studies were performed as described previously. Cells (5 × 10^6) were harvested at the end of the 7-day culture period, washed twice with serum-free RPMI medium, once with binding buffer, and acidified with CO2 (gas). Cell pellets were resuspended in binding medium and aliquoted into siliconized (Sramcatoe, Sigma) Eppendorf tubes. In cold-competition studies, cells were preincubated with 25- to 50-fold excess of unlabeled TGF-β, at 4°C for 10 minutes. All tubes received 1 × 10^6 cpm of [3H]-TGF-β, (specific activity 100 to 140 μCi/μg; Biomedical Technologies, Stoughton, MA) and were incubated for 30 minutes at 4°C. Cell pellets were then washed and resuspended in cross-linking buffer containing 100 μg/mL disuccinimidyl suberate (Pierce Chemical, Rockford, IL) and rotated for 30 minutes at 4°C. Cells were subsequently washed twice with washing buffer, and the cell pellet was lysed. Cell lysates were subjected to electrophoresis under reducing conditions using a 7% to 15% gradient gel. Autoradiograms were obtained after exposure of gels to Kodak X-OMAT film for 7 days at -70°C.

**Quantitation of TGF-β steady-state mRNA expression.** For quantitation of total mRNA expression, 2 × 10^5 HL-60 cells were harvested after treatment as indicated in the legends to Figs 1 through 5, washed twice with phosphate-buffered saline (PBS) and lysed with 4 mol/L guanidine thiocyanate solution. RNA was isolated through CsCl gradient ultracentrifugation (32,000 rpm, 16.5 hours) and total RNA was ethanol-precipitated. The amount of RNA was determined using spectrophotometric analysis at 260 nm. Equal loading of sample and transfer of RNA was verified by ethidium bromide staining of the agarose gels before and after transfer to nitrocellulose membranes.

For Northern blot analysis, 10 μg total RNA or 15 μg poly-A (+) mRNA was loaded per lane, electrophoresed using 1% agarose-formaldehyde gels, transferred to nitrocellulose membranes, and prehybridized overnight at 42°C. Northern blots were hybridized overnight at 42°C with 32P-labeled 1.6-kilobase (kb) cDNA probe for mouse TGF-βs, 2.35-kb cDNA probe for human TGF-βs, and 2.2-kb cDNA probe for chicken TGF-βs. Northern blots were washed for 30 minutes at room temperature using 2× SSC and 0.1% SDS solution. Autoradiograms were obtained after exposure of blots to Kodak X-OMAT film at -70°C with intensifying screens. RNA from CT6, NRK, and PC3 cell lines served as positive controls for TGF-βs, TGF-βs, and TGF-βs mRNA expression.

**Quantitation of active TGF-β protein in culture supernatants.** Culture supernatants from RA-treated HL-60 cells were tested for 3 days with various concentrations of RA, harvested, and frozen. Active TGF-β protein in the supernatants was determined directly using colony formation of NRK 49F cells in soft agar as described.
Cold TGF-β

<table>
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<th>0.1 nM RA</th>
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<td>200,000</td>
<td>100,000</td>
<td>68,000</td>
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Fig 2. Affinity labeling of TGF-β1 receptors on RA-treated HL-60 cells. After HL-60 cells were treated with various concentrations of RA for 7 days, 5 × 10⁶ cells were preincubated with or without unlabeled TGF-β1 (40 ng/mL). Cells were then incubated with 1 × 10⁶ cpm ¹²⁵I-TGF-β1, cross-linked by disuccinimidyl suberate (100 μg/mL), lysed, and subjected to electrophoresis through a 7% to 15% gradient gel under reducing conditions. The autoradiogram was made after 7-day cell exposure at -70°C with intensifying screens.

Previously, twelve-well tissue culture dishes were coated with 0.5% Noble agar (0.4 mL, Difco, Detroit, MI). NRK-49F cells (0.2 mL of a suspension of 3 × 10⁶ cells/mL) were mixed with 0.6 mL Noble agar and 0.2 mL sample to which 5 ng/mL epidermal growth factor (BRL, Bethesda, MD) had been added. The NRK-49F cell suspension (0.4 mL) was then pipetted onto the agar underlay and incubated for 7 days at 37°C. After incubation, 0.4 mL of p-iodonitrotetrazolium violet dye (0.5 mg/mL, Sigma) was added to each well and incubated for 18 hours longer. Colonies (>100-μm diameter) were visualized and counted. The activity in the culture supernatants was quantified by interpolation of colony numbers on the dose-response curve generated using purified TGF-β.

RESULTS

Effects of TGF-β on proliferation of RA-treated HL-60 cells. Consistent with previous studies, treatment of HL-60 cells with increasing concentrations of RA resulted in a dose-dependent decrease in proliferation. Maximum inhibition occurred after culture of HL-60 cells with 10 to 100 nmol/L RA for 7 days (Fig 1). Further incubation of 10 to 100 nmol/L RA pretreated HL-60 cells with exogenous TGF-β did not result in any further decrease in proliferation. Neither did treatment of HL-60 cells with TGF-β result in proliferation of cells cultured in medium or 0.1 nmol/L RA. In contrast, TGF-β dose-dependently inhibited proliferation of HL-60 cells pretreated with a suboptimum concentration of RA (1.0 nmol/L; Fig 1).

Effects of TGF-β on differentiation state of RA-treated HL-60 cells. To determine the effects of TGF-β on HL-60 cell differentiation, cells were cultured for 7 days with or without RA, cultured with TGF-β for 3 more days, and then assayed for differentiation. Morphologic and cytochemical evaluation of RA-treated HL-60 cells showed a dose-dependent increase in the percentage of granulocytic cells (Table 1). Subsequent addition of TGF-β to these cultures did not significantly alter granulocytic differentiation of the HL-60 cells relative to RA-treated control cultures even in cases in which TGF-β addition resulted in an antiproliferative state. At high concentrations of RA, addition of exogenous TGF-β resulted in a slight increase in monocytic cell differentiation. These data confirm that high concentrations of RA resulted in increased granulocytic differentiation and show that TGF-β had only slight effects on differentiation of RA-treated HL-60 cells.

Induction of TGF-β receptor expression of HL-60 cells by RA. To determine whether the enhanced sensitivity of RA-treated HL-60 cells to the antiproliferative effects of TGF-β resulted from increased expression of the receptor for TGF-β, we examined cell surface receptor expression by chemical cross-linking. TGF-β binds to three receptor types and results in formation of three cross-linked complexes: type I, 65 Kd; type II, 85 to 110 Kd; and type III, 250 to 350 Kd proteoglycan-rich binding complex. Unstimulated HL-60 cells expressed low levels of the 65-Kd TGF-β receptor, which increased in a dose-dependent manner after treatment with RA. Increased TGF-β receptor expression was observed after 3-day treatment with RA (data not shown). Maximum receptor expression occurred after 7-day culture with 10 nmol/L RA with predominant expression of the 65-Kd receptor (Fig 2).

To determine whether TGF-β receptor induction was restricted to RA-induced granulocytic differentiation, a variant of the HL-60 cell line that undergoes monocytic
Expression of steady-state levels of TGF-β mRNA levels in RA-treated HL-60 cells. Because treatment of HL-60 cells with RA increased TGF-β, receptor expression (Fig 2) and resulted in sensitivity to the antiproliferative effects of TGF-β, (Fig 1), we wished to determine whether RA also induced TGF-β mRNA expression during HL-60 cell differentiation. Total mRNA extracted from HL-60 cells demonstrated a dose-dependent induction of the 2.5-kb TGF-β, mRNA species after RA treatment. Maximum induction of the 2.5-kb mRNA species occurred after treatment with 10 nM RA (Fig 4). The constitutive expression of a 4.1-kb mRNA species that hybridized to the TGF-β, cDNA probe varied between experiments and was greatly reduced after selection for poly-A (+) mRNA. HL-60 cells also expressed constitutive levels of a 4.3-kb TGF-β, poly-A (+) mRNA species, whereas no TGF-β, poly-A (+) mRNA was detectable (Fig 5). In contrast to the 2.5-kb, TGF-β, mRNA species, treatment with RA did not modulate TGF-β, or TGF-β, mRNA expression.

Quantitation of TGF-β activity in HL-60 culture supernatants. Because many cell types have been shown to express TGF-β, mRNA and to express the protein in a latent (inactive) form, we performed studies to determine whether active TGF-β protein was being produced in HL-60 cultures. Culture supernatants were harvested from HL-60 cells treated for 3 days with various concentrations of RA and assayed for the presence of proteins with TGF-β-like antiproliferative activity (Table 2). In two separate experiments, medium-treated HL-60 cells did not produce active TGF-β. Treatment with RA resulted in a

- 4.4 kb
- 2.1 kb

Fig 4. Steady-state TGF-β, mRNA expression in RA-treated HL-60 cells. Total RNA was isolated from 2 x 10⁶ HL-60 cells after 7-day treatment with various concentrations of RA. Northern blot analysis of 10 μg per lane of RNA is shown after hybridization with a ³²P-labeled cDNA TGF-β, probe. The autoradiogram was made after 2-hour exposure at -70°C with intensifying screens.
production of recoverable active protein. Thus, RA treatment resulted in an increase in both TGF-β, mRNA expression (specifically the 2.5-kb species) and an increase in protein with TGF-β-like activity.

**DISCUSSION**

The mechanism by which RA induces differentiation of HL-60 cells is unknown, but numerous reports have indicated that several cytokines, alone or in combination, can induce HL-60 cell differentiation. Many of these studies

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### Table 1. Morphologic and Cytochemical Analysis of HL-60 Cells After RA and TGF-β, Treatment

<table>
<thead>
<tr>
<th>RA (nmol/L)</th>
<th>TGF-β,</th>
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<th>Granulocyte Differential</th>
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<td></td>
<td></td>
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<td>ANAE (%)</td>
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<td>12.0</td>
<td>61</td>
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</table>

Cells (2 x 10⁶/mL) were cultured and assayed as described in the Materials and Methods section. HL-60 cells were treated with RA for 7 days and subsequently treated with or without TGF-β, (10 ng/mL) for an additional 3 days in culture.

*Mature cells are representative of the percentage band and segmented forms of granulocytes.

### Table 2. TGF-β Activity in Culture Supernatants From HL-60 Cells Treated for 3 Days With or Without RA

<table>
<thead>
<tr>
<th>Treatment Medium*</th>
<th>TGF-β Activity (pg/mL)†</th>
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<tr>
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<td>10.0</td>
<td>56</td>
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<tr>
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<tr>
<td>Experiment 2 (nmol/L RA)</td>
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<td>10.0</td>
<td>140</td>
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*HL-60 cells were cultured for 3 days with various concentrations of RA. Culture supernatants were harvested and tested for TGF-β activity.
†Culture supernatants were assayed by colony formation in soft agar using NRK-49F cells.
have used known cytokines such as TNF and interferon as inducers of HL-60 monocytic cell differentiation. Recently, the finding that TGF-β synergizes with TNF to induce HL-60 monocytic cell differentiation established a role for TGF-β in development of hematopoietic cells apart from the previously described inhibitory role of TGF-β. Our data support a role of TGF-β during hematopoietic cell differentiation and demonstrate that cessation of proliferation can occur independent of acquisition of a terminally differentiated phenotype.

Our studies show that HL-60 cells cultured in the absence of RA were not sensitive to the antiproliferative effects of exogenous TGF-β. HL-60 cells cultured under conditions suboptimum for differentiation (1.0 nmol/L RA), were sensitive to the antiproliferative effects of TGF-β, however, and showed no marked change in cell differentiation in response to TGF-β. One mechanism by which this sensitivity to TGF-β may occur is modulation of functional TGF-β receptor expression. Cross-linking studies demonstrated that medium-treated HL-60 cells expressed low levels of the 65-Kd receptor species that increased on treatment with as little as 1.0 nmol/L RA. This concentration of RA also induced HL-60 cells sensitive to the actions of exogenous TGF-β. The induction of TGF-β receptor expression on HL-60 cells by RA was observed as early as 3 days but was maximum by 7 days (data not shown). These data indicate that treatment of HL-60 cells with suboptimal concentrations of RA results in upregulation of functional TGF-β receptors.

The ability to increase TGF-β, binding after RA treatment was also observed in the HL-60 variant HL-60/MR1 during programmed monocytic cell differentiation. The RA concentration required for maximum receptor induction was lower than that observed in the parental HL-60 cells. Furthermore, HL-60/MR1 cells exhibited maximum receptor induction by 3 days, in contrast to the parental HL-60 cell line that required 7 days. These findings are consistent with the previous report which showed increased sensitivity to RA during HL-60/MR1 monocytic differentiation. Treatment with RA resulted in an increase in expression of all three receptor types on HL-60/MR1 cells, whereas a predominant increase in expression of the 65-Kd binding protein was observed on parental HL-60 cells. The induction of multiple TGF-β receptor types was also observed during monocytic differentiation of parental HL-60 cells in response to TNF-α.

Previous studies have shown that RA treatment had no effect on TGF-β receptor expression on NRK cells as measured by binding assays. In contrast, Rizzino reported that RA treatment of embryonal carcinoma cells increased TGF-β receptor expression. Our data support this latter finding of RA induction of TGF-β receptor expression. These reports indicate that RA induction of TGF-β receptors could be restricted to certain cell types. Differences owing to environmental conditions of the cell or differential sensitivity of the binding technique cannot be ruled out, however.

RA has also been shown to affect other aspects of the TGF-β system aside from expression of its receptor. Recent studies by Glick et al showed marked increases in TGF-β mRNA expression and protein production during murine keratinocyte differentiation in response to RA and Ca²⁺. In contrast to the studies by Glick et al, our data show that RA treatment of HL-60 cells increased expression of the 2.5-kb mRNA transcript for TGF-β, and had no effect on the level of TGF-β mRNA expression. The ability of RA to increase steady-state levels of TGF-β mRNA species was apparent by 3 days of culture (data not shown) and maximum by 7 days of culture and allowed sufficient production and secretion of active TGF-β-like proteins to be measured by NRK 49P cell colony formation in soft agar.

Our findings support the hypothesis of a two-signal model for cell differentiation. The first signal is a "priming" signal that results in increased production of negative growth regulators, such as TGF-β, which halt proliferation of the cell. Indeed, we showed that RA induces expression of both functional TGF-β receptors and production of active TGF-β-like protein. The requirement for a second signal in terminal differentiation of HL-60 cells is supported by the absence of differentiation during growth inhibition by exogenous TGF-β. The identity of these differentiation signals that program either granulocytic differentiation in HL-60 cells or monocytic differentiation in HL-60/MR1 cells is unknown, but the ability of TNF-α to synergize with TGF-β in monocytic differentiation of HL-60 cells may provide insight regarding one potential second signal for differentiation.

Induction of both the TGF-β receptor and active TGF-β-like protein after treatment of HL-60 cells with RA suggests that induction of a TGF-β-mediated antiproliferative autocrine loop occurs during differentiation of HL-60 cells. To determine whether the TGF-β-mediated antiproliferative signal is required during RA-induced differentiation of HL-60 cells, anti-TGF-β antibodies were included during treatment of HL-60 cells with RA. Examination of the effects of anti-TGF-β on the proliferation and differentiation of HL-60 cells has been problematic, however. TGF-β was shown previously to bind to a number of serum carrier proteins, and recent studies have shown that anti-TGF-β antibodies can block the effects of endogenous TGF-β only under serum-free conditions. However, HL-60 cells grown under these conditions undergo increased spontaneous differentiation and cessation of proliferation during the 7-day culture period (unpublished observations).

Because RA treatment for 7 days is required for maximum differentiation in the presence of serum, the establishment of serum-free conditions for the anti-TGF-β studies has proven difficult. Studies are continuing in examination of the role of TGF-β in an autocrine negative feedback loop during RA-induced differentiation of HL-60 cells.

RA has been used successfully to treat patients with acute promyelocytic leukemia and has been shown to induce terminal differentiation of primary acute promyelocytic leukemic cells in vivo and in vitro. Induction of a TGF-β autocrine inhibitory loop by RA could play a role in the subsequent terminal differentiation of these cells. Our
studies suggest that combination therapy of RA and TGF-β may provide additional therapeutic benefit. In addition, studies have shown that RA treatment could induce growth inhibition of certain human melanoma and breast carcinoma cells lines and that TGF-β may be an autocrine growth inhibitor of estrogen-receptor-negative human breast cancer cell lines. Thus, in a number of malignancies, RA and TGF-β may be useful as potential therapeutic agents in induction of tumor cell growth inhibition and differentiation.

ACKNOWLEDGMENT

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REFERENCES

31. Oliver-McCourt MD, Wakefield LM: Latent transform-
EFFECTS OF TGF-β ON RA-TREATED HL-60 CELLS


LA Falk, F De Benedetti, N Lohrey, MC Birchenall-Roberts, LW Ellingsworth, CR Faltynek and FW Ruscetti