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RESEARCH ARTICLE

Biology of the Opioid Growth Factor – Opioid Growth Factor Receptor Axis: Bench to Bedside and Back

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ABSTRACT

The Opioid Growth Factor – Opioid Growth Factor Receptor axis was identified nearly 30 years ago in our laboratory when we demonstrated that an endogenous opioid pentapeptide had inhibitory growth activity following injection into normal animals. In addition to validating this action for the peptide, identification, characterization and cloning of the specific receptor related to the opioid growth factor, chemically termed [Met^5]-enkephalin, revealed that the receptor was unique in its biochemical and molecular structure, but had pharmacological properties similar to other opioid receptors. Mechanisms associated with binding of the opioid growth factor to its receptor located on the outer nuclear envelop and the transport into the nucleus were identified. The selectively of the peptide for the receptor was demonstrated in normal and cancer cell lines. This uniqueness of the peptide-receptor interaction was evidenced by the duration of receptor blockade in that intermittent blockade resulted in decreased cell replication and complete blockade resulted in accelerated proliferation. Investigations on the mechanism of action, as well as the dysregulation of the Opioid Growth Factor – Opioid Growth Factor Receptor axis in human pathology, have resulted in bench to bedside and bedside to bench discoveries. Receptor blockade by naltrexone has been used clinically for treatment of fatigue and other immune deficiencies in many autoimmune disorders. The research has gone from bench to bedside and has returned to the bench for additional investigation of mechanisms driving the action of the axis and resultant function following receptor blockade.

Keywords: [Met^5]-enkephalin, diabetes, dry eye, multiple sclerosis, low-dose naltrexone

1. Introduction

Nearly three decades have passed since the discovery of the Opioid Growth Factor (OGF), chemically termed [Met^5]-enkephalin. This endogenous pentapeptide is both a neurotransmitter and growth modulator, and has multiple binding sites. The role of OGF that is of interest in our laboratory has been that of cellular replication. Opioid growth factor regulates cell replication by binding to a novel nuclear-envelope associated receptor termed OGFr. Together the peptide and receptor maintain cellular homeostasis. The OGFr was initially termed the zeta opioid receptor (ζ) but renamed when the primary agonist ligand was determined to be OGF. The receptor has been identified, isolated, characterized and cloned in rat, mouse, and human[1]. Since the cloning of the receptor in 2000, mechanisms of transport of the ligand and receptor into and out of the nucleus have been determined. Importantly, our laboratory has focused on dysregulation of the peptide-receptor pathway and effects on diabetes, cancer, and autoimmune disorders. Investigations began at the laboratory bench where the mechanism of transport and action were defined, then moved into the clinical realm as it was discovered that the dysregulation of the Opioid Growth Factor – Opioid Growth Factor Receptor axis was associated with human pathology. Preclinical studies on receptor blockade by naltrexone have been translated to the clinic for treatment of fatigue and other immune deficiencies in many autoimmune disorders. The research has gone from bench to bedside and has returned to the bench for additional investigation of mechanisms driving the action of the OGF-OGFr axis in both the normal and pathologic state.

2. Identification of the OGF-OGFr Pathway

In the early 1980s an endogenous opioid peptide termed methionine enkephalin was discovered to have inhibitory growth properties in addition to those of neurotransmission[2-5]. This pentapeptide inhibited cell replication in normal brain tissue[2,3], human cancer cell lines and nude mouse tumors[4]. The peptide was immunohistochemical identified in brain tissue and shown to alter DNA synthesis in a wide variety of organs and tissues[6]. To distinguish this activity from the role of neurotransmission, the peptide was termed Opioid Growth Factor. Because of its action *in vitro* it was hypothesized that there was a unique receptor mediating peptide activity. Extensive investigations confirmed the presence and function of a nuclear membrane associated receptor[1]. Immunohistochemical and immunoelectron microscopy studies showed that the receptor was located on the outer nuclear envelope[7]. Biochemical and pharmacology studies revealed that the specific agonist-ligand for this receptor was the opioid growth factor.

The gene for human OGFr is 9 kb consisting of 7 exons and 6 introns. The human gene codes for a 677aa protein that includes several imperfect repeats of 20 amino acids each and 3 nuclear localization signals[1]. The OGFr has a mass of 62kD and is located on chromosome 20q13.3 in humans[1].

Because OGFr has classical opioid receptor characteristics such as stereospecificity and naloxone reversibility it was originally named zeta (ζ) to be consistent with the μ, Δ, and κ classical opioid receptors discovered years

earlier. However, molecular studies on the genetic structure confirmed that OGFr had no homology and little overlap at the amino acid level with classical opioid receptors. Molecular isolation and characterization identified the genetic and protein sequences in rat, mouse, and human. The lack of significant homology to known domains or functional motifs of the μ, Δ, and κ opioid receptors supported the name change from ζ to OGFr[1].

2.1 MECHANISMS OF ACTION

The OGF-OGFr axis is involved in cell replication. Electron microscopic studies confirmed that OGFr is localized on the outer nuclear envelope[7]. Investigations were undertaken to determine how the OGF agonist reaches the internalized receptor[8]. Using 5,6-tetramethylrhodamine OGF the temporal course of cellular uptake was monitored. Internalization of the labeled probe was both temperature and time dependent. Using an *in vitro* model in which COS-7 cells were incubated with labeled OGF, the OGF was detected in the cytoplasm within 15 minutes and inside the nucleus within 30 minutes[8]. The OGF label lasted for 5 hours. The addition of clathrin siRNA diminished uptake of RhoOGF and also decreased DNA synthesis. Proliferation was modified by the internalized labeled OGF allowing the conclusion that OGF is actively internalized by way of clathrin-mediated endocytosis and acts to down-regulate cell synthesis[8].

The next question was how the receptor-agonist complex interacted at the nuclear membrane. Nucleocytoplasmic trafficking of the OGF-OGFr complex is dependent on nuclear localization signals[9]. Using the full-length OGFr fused with enhanced green fluorescent protein, the eGFP probe indicated that translation of OGFr occurred in 8.5 hours, required 8 hours to transit into the nucleus and remained intact for 8 days. The OGFr was visually followed by fluorescent microscopy from the outer nuclear envelope to the paranuclear cytoplasm and into the nucleus. Migration into the nucleus occurred via nuclear localization signals (NLS), of which there were three sequences that served as localization signals. Simultaneous mutation of $NLS_{383-386}$ and $NLS_{456-460}$ abolished movement of eGFP-OGFr suggesting that they were necessary for transit. It was determined that an intact OGFr had to be localized inside the nucleus for cell proliferation to occur[9].

The specific transport factors required for the OGF-OGFr complex to transit the cytoplasm to the nucleus were investigated using genetic methodology in an *in vitro* setting. Squamous cell carcinoma of the head and neck cells (SCC-1) were transfected with siRNAs in order to study the role of karyopherin α1, α2, α3, α4, or β1 or Ran. SCC-1 cells were treated with each karyopherin siRNA individually as well as mixtures, and it was noted that cells with karyopherin β1 and Ran did not allow transport of the eGFP labeled OGFr to move into the nucleus[10]. Functional studies to monitor the level of DNA synthesis revealed that cells with siRNA (knockdown) of β1 and Ran had more BrdU labeling than in cultures with single karyopherin knockdowns. Thus, karyopherin β1 and Ran are necessary for the import of the OGF-OGFr complex into the nucleus for the proper replicative function[10].

The mechanistic pathway by which OGF modulates DNA synthesis is via the p16 or p21

cyclin-dependent inhibitory kinases. The primary function of the OGF-OGFr regulatory pathway is to maintain cellular homeostasis by inhibiting cell proliferation and retarding progression through the cell cycle. This evidence was documented in head and neck cancer cells, pancreatic cancer cells, normal human umbilical vein endothelial cells, and human epidermal keratinocytes[11-13]. The mechanism of the OGF interaction with OGFr in both normal and a wide-variety of cancer cells implicated DNA synthesis. In squamous cell carcinoma only the $p16^{INK4a}$ cyclin dependent inhibitory kinase is present and thus OGFr can downregulate cell proliferation utilizing this pathway alone[11]. In pancreatic cancers that lack p16, the $p21^{WAP1/CIF1}$ pathway is selected[12]. In normal cells both pathways are intact and both are utilized. Thus, the OGF-OGFr axis regulates the G_1/S interface of the cell cycle through the cyclin-dependent inhibitory kinases by delaying transit through the cell cycle[13]. Programmed cell death and/or differentiation processes do not seem to be impacted by the OGF-OGFr axis.

2.2 NALTREXONE BLOCKADE OF THE OGF-OGFR PATHWAY

One of the most prominent characteristics of the OGF-OGFr pathway is the ability to pharmacologically block this receptor using opioid receptor antagonists such as naloxone and naltrexone. Naltrexone is a synthetic opioid developed in the late 1960s for blockade of mu opioid receptors. In 1984 it was approved by the FDA for treatment of opioid addiction at high dosages (≥ 50 mg per tablet) that block the classical opioid receptors in a continuous manner[14]. A decade later, Revia© was approved for treatment of alcohol dependency[14]. The low profile of risk to benefit ratio enhanced the use of naltrexone as a first-line treatment of opioid use disorder and alcohol use disorder.

Preclinical studies using escalating doses of naltrexone demonstrated that the higher the dosage the more cell proliferation occurred, rather than the expected phenomenon of higher doses resulting in greater cellular inhibition. Studies were conducted in a variety of tissues to reproduce the results that the magnitude and direction of effects were based on the duration of OGFr blockade and not dose response[15]. This biphasic property is not unique in the world of biology, but was probably the most important aspect of the OGF-OGFr axis and led to several fortuitous scenarios. The most potent antagonist of OGFr appears to be naltrexone, an FDA approved treatment of addiction, alcoholism, and obesity. The FDA approval renders the use of naltrexone in any other form as a "repurposed" drug[14]. Because of the divergence in dose-response actions, research has extended into two different directions depending on the length of receptor blockade. Intermittent OGFr blockade that last less than 12 hr per 24 hr cycle results in an elevation of the autocrine and/or paracrine-produced OGF and exerts an inhibitory action on cell replication. Total blockade of OGFr that last at least 20 hr per 24 hr cycle and is usually established by higher doses of naltrexone, or multiple administrations of low doses, per day resulted in enhanced cell proliferation.

2.3 MECHANISM OF NALTREXONE BINDING

Following the demonstration that naltrexone was a potent opioid antagonist that altered

cell proliferation, it was imperative to investigate how naltrexone entered cells, passed through the nuclear membrane and interacted with OGFr residing on the outer nuclear envelope[16]. Naltrexone is a small molecule, ~377 MW, and was fluorescently tagged with [1-(N)-fluorosceinyl naltrexone thiosemicarbazone] to create F-naltrexone. Cells grown *in vitro* were bathed in F-naltrexone and beginning at 1 min of incubation, live-cell images were taken to monitor the movement of F-naltrexone. Deconvoluted microscopy enabled us to detect the label in a variety of cell lines including COS-7 African Green monkey, human mesenchymal stem cells, human head and neck squamous carcinoma cells, and MIA PaCa-2 human pancreatic cancer cells. A comparable temporal-spatial distribution was recorded by microscopy. Within minutes the F-naltrexone passively diffused into the cells and remained internalized for up to 48 hr. Diffusion occurred at 4°, 22°, and 37°C. There was no competition when other opioid antagonists or ligands were added to the cultures; however, fluorescein alone did not enter the cells suggesting that the catalyst was in fact naltrexone. The efficacy of F-naltrexone to alter DNA synthesis was not substantially reduced such that BrdU synthesis was increased in F-naltrexone treated cells relative to vehicle-treated cell cultures and comparable to cultures treated with unlabeled naltrexone[16].

3. Translational Studies: Down-regulated OGF and/or OGFr

Recognition that the duration of receptor blockade determined the function of the OGF-OGFr pathway led to many preclinical studies related to different pathologies.

3.1. DOWN-REGULATION OF OGF AND OGFR IN CANCER

As noted, multiple routes of dysregulation of the OGF-OGFr pathway could occur. OGF levels in plasma and/or tissues could be up-regulated or down-regulated with OGFr remaining intact, or OGFr function could by dysregulated along with variable OGF expression. Our research team began their investigation of dysregulation by examination of the translation of OGF-OGFr axis as a physiological determinant of cell proliferation in cancer. Assuming that nearly all cancers are rapidly dividing cells, the role of OGF as a tonic, stereospecific, noncytotoxic, and nonapoptotic pentapeptide was examined in 31 human cancer cell lines[17].

Human cancer cell lines representative of 20 different systems within the human body were examined for the presence of OGFr using molecular technology. Three human cell lines were studied in detail – SCC-1 head and neck squamous carcinoma, MIA PaCa-2 pancreatic cancer, and KAT-18 thyroid cancer. OGFr was detected in all cell lines and if cells were treated with an OGFr siRNA, there was less receptor; the amount of knockdown was variable with KAT-18 showing less than 20% of control levels 48 hr after treatment[17]. Inhibitory effects of OGF and enhanced growth effects of naltrexone were demonstrated. The specificity of OGF as the inhibitory ligand of choice was demonstrated. The ubiquity of the OGF-OGFr axis in 31 human cell lines warrants continuing our bedside- and bench-studies to determine mechanisms and pathways utilized by the OGF-OGFr axis in the regulation of cancer cell growth. Information related to the presence of the regulatory axis may be of importance in cancer diagnosis and treatment design.

Mutations in OGFr in several human cancers alter the function of OGFr following OGF binding[18]. It is known that the OGF-OGFr axis tonically modulates cancer growth and that OGF acts as an autocrine-produced inhibitory pentapeptide. For OGF to be effective it must bind to OGFr and together they traffic into the nucleus where OGF suppresses the cell cycle. To validate the required integrity of OGFr, missense mutations in OGFr that were documented in the Catalogue of Somatic Mutations in Cancer were the focus of *in vitro* assays. A functional assay using 5-bromo-2-deoxyuridine (BrdU) revealed that if OGFr is mutated, OGF will not bind and together OGF-OGFr will not transit into the nucleus. This information led to additional preclinical studies that examined nuclear export mechanisms of OGFr[19]. Once the receptor is inside the nucleus, does it degrade or return to the cytoplasm? Molecular studies demonstrated that labeled OGFr accumulated in the nucleus if CRM-1 was abrogated. If the export sequence of CRM1 was mutated, OGFr stayed inside the nucleus suggesting that OGFr moved out of the nucleus in a CRM1 dependent manner[19].

3.2. MOLECULAR STUDIES ON EXPRESSION LEVELS OF OGFR IN CANCER

Tissue culture studies using several lines of human ovarian cancer that were transfected to either over-express or under-express OGFr substantiated the findings that OGF acts through this receptor to either decrease or increase cell proliferation, respectively[20,21]. SKOV-3 ovarian cancer cells were molecularly engineered to under-express OGFr. Two clonal lines containing shRNA OGFr constructs had protein decreases of approximately 70%. Binding assays revealed reductions in OGFr receptor binding by 50% or more; binding affinity was normal indicating that only the quantity of OGFr receptor was diminished. The lack of OGFr resulted in increased DNA synthesis in the clonal lines for more than 130% relative to wildtype and empty vector cell lines. Inoculation of the transfected cells into nude mice resulted in increased tumor growth, and the addition of OGF which normally reduced tumor size and growth, had no effect suggesting that the molecular manipulation of receptor number to a subthreshold value is a critical determinant of the progression of ovarian cancer[21]. Use of siRNA technology to silence OGFr stimulated cancer cell replication. Further molecular manipulation to knockdown p16 or p21 in OVCAR-1 human cancer cells eliminated the ability of OGF to inhibit growth *in vitro*. Nude mice injected with cells lacking a full complement of OGFr developed tumors earlier and larger than when tumor cells contained empty vectors[21].

In another series of experiments, the duration of blockade was shown to predict the effect on growth utilizing human ovarian cancer cells in vitro[22]. Intermittent doses of naltrexone upregulated the expression of peptide and receptor at the translational level but had no effect of transcription. Inhibited cell replication required the presence of p16 or p21 cyclin-dependent inhibitory kinases. No evidence of apoptosis or necrosis was noted[22].

In summary because LDN is effective in the treatment of cancer and the molecular studies showing that disruptions in the OGFr pathway led to increased growth, it appears that cancer has decreased expression of OGFr,

thus requiring that more OGF peptide be available for the low receptor number for OGF to be therapeutically effective.

4. Opioid Growth Factor in Autoimmune Disorders: Clinical studies

In the early 2000s, conversations occurred between our laboratory and that of Dr. Bihari, an eminent physician in New York. We relayed our observations that there was inhibited cell proliferation following administration of low doses of naltrexone. Dr. Bihari capitalized on these conversations and began using low doses of the compound for treatment of HIV/AIDS which at the time was an uncontrollable disease and notable patients were succumbing[23]. The name LDN (i.e., low-dose naltrexone) was coined. The success of LDN to restore the immune system and activate anti-inflammatory cytokines to combat AIDS[23] resulted in an explosion of research articles related to the use of naltrexone for treatment of diseases other than addiction and alcoholism.

4.1. DOWN-REGULATION OF OPIOID GROWTH FACTOR IN PATIENTS WITH AUTOIMMUNE DISEASES

In 2023, results from several clinical trials on pain management and/or fibromyalgia reported that LDN had few, if any, side-effects, and was effective at reducing pain[24,25]. In all cases, including small case studies, the authors suggested that large, randomized, controlled clinical trials were needed. Yang[24] carried out a meta-analysis and systematic review of 805 articles that included the term naltrexone; a final group of 9 articles were evaluated. No severe adverse events were reported, and while efficacy was apparent, scientific proof of efficacy still needs to be addressed in larger trials. The biphasic dose response relationship is evident with naltrexone as a treatment of pain as low doses inhibit pain and higher doses are stressful and increase pain. Driver and D'Souza[25] reported on a 14-year retrospective study on the use of LDN for fibromyalgia and pain and stated that LDN did not harm and was effective for some patients. Dara and colleagues reviewed publications on pain and LDN and concluded that LDN provides relief in chronic inflammatory conditions such as fibromyalgia, multiple sclerosis, and Crohn's disease[26]. All authors concluded that LDN treatment of pain associated with other immune disorders is beneficial and warrants further study related to methods of weaning patients off traditional therapies and onto LDN.

Srinivasan and group[27] reported on the results from a study on chronic diabetic neuropathy where patients received either LDN or amitriptyline. Researchers concluded that LDN had comparable efficacy to the standard therapy amitriptyline, but had a superior safety profile with only 8 adverse effects recorded with LDN, most of which were diarrhea in comparison to 52 adverse events reported with amitriptyline[27].

Particularly during the COVID-19 pandemic there was a resurgence of homecare remedies. The use of LDN offered the advantages of being safe, inexpensive and an oral therapy. The presumed action of controlling the cytokine storm associated with COVID was a positive factor. Isman[28] reported that LDN at the upper end of the low dose range (i.e., 4.5 mg) plus NAD+ patches were effective for reducing persistent fatigue. Side

effects were reduced or eliminated by adjusting dosages, and offered the advantages of being inexpensive and available orally.

Bonilla and colleagues[29] carried out a retrospective review of a small cohort of patients who had received LDN off-label as a therapeutic for post-acute sequela from COVID. Low dose naltrexone treatment appeared to improve clinical symptoms of fatigue and abnormal sleep, had few recorded adverse events, and persons receiving LDN had better functional status. In summary evidence, even if circumstantial, purports that intermittent blockade of OGFr, alone or with other receptors, had beneficial effects for pain relief, fatigue, and symptoms of autoimmune disorders.

Our laboratory reported that intermittent blockade of the OGFr using LDN reduced anxiety and altered fatigue levels in persons with multiple sclerosis, with or without COVID[30,31]. Data from surveys revealed that, despite the study being underpowered, those taking LDN either alone or in combination with other standard therapies had significantly lower self-reported anxiety and depression scores in comparison to persons with multiple sclerosis and not taking LDN[30]. The length of time that a person had the MS diagnosis was related to anxiety/depression. The longer someone had MS, the less anxiety and/or depression was reported[31]. The length of disease regardless of the age of onset determined levels of perceived anxiety and/or depression.

4.2. DOWN-REGULATION OF THE OGF-OGFR IN AUTOIMMUNE DISORDERS: PRECLINICAL STUDIES

Our preclinical investigations began with experiments that measured proliferation of T lymphocytes[32] and B lymphocytes[33]. Using splenic-derived T cells that were stimulated by phytohemagglutin (PHA), immunoreactivity of OGFr and OGF was detected in culture[32]. Stimulated vehicle-treated cells showed the presence of reactivity, but cells grown in the presence of OGF were markedly suppressed in a dose-dependent manner with concomitant decreases in DNA synthesis. The PHA-stimulated cells were not affected by other natural and synthetic opioid-related compounds. The addition of NTX to consistently block the cells had no effect. Likewise, antibody neutralization or non-stimulated T cells resulted in no response to the addition of OGF. Molecular studies confirmed that the downregulation of PHA-stimulated T cells was via the OGFr axis. Other cell lines expressing classical opioid receptors (e.g., μ and Δ) transfected with receptor-specific siRNAs were not altered in cell number by the addition of OGF. The studies confirm that the OGF-OGFr axis is present in T lymphocytes and acts as an immunosuppressant.

B lymphocytes from mice that were stimulated to divide by lipopolysaccharide (LPS) had inhibited proliferation following exposure to several dosages of OGF[33]. Within 72 hr, cell number was inhibited more than 40% by OGF whereas no other endogenous or exogenous opioid had an effect on growth. If classical opioid receptors were down-regulated by siRNAs, OGF had no effect on proliferation. In summary, these studies demonstrated that the OGF-OGFr axis is present and functioning in stimulated T- and B-cells, and the system plays a role in modulating autoimmune diseases.

With this understanding of immune responses, preclinical studies utilizing mice receiving

myelin oligodendrocytic glycoprotein (35-55) to induce experimental autoimmune encephalomyelitis were treated from the time of induction with high dose naltrexone (10 mg/kg NTX), low dose naltrexone (0.1 mg/kg) or vehicle. Mice with experimental autoimmune encephalomyelitis and receiving either high doses of naltrexone or vehicle daily had comparable pathologies suggesting that complete blockade of the OGFr daily did not enhance neurological deficits observed in the mice with experimental autoimmune encephalomyelitis. However, one—third of mice receiving LDN did not exhibit behavioral signs of disease and those with disease symptoms were markedly reduced. Fewer activated astrocytes and less demyelination were observed in the LDN cohort, implying that endogenous opioids evoked by LDN and acting during the rebound period after drug exposure were inhibitory to the onset and progression of experimental autoimmune encephalomyelitis[34-37].

Researchers in the field of autoimmune disorders have capitalized on intermittent blockade of opioid receptors and the use of low doses of naltrexone or very low doses of naltrexone for treatment of autoimmune disorders. However, for LDN to be effective, opioid receptors, and presumably OGFr need to be intact. At this time, the mechanism of action is not confirmed to be solely OGFr, but could in fact be modified by other opioid receptors such as mu opioid receptor[38], or even non-opioid receptors such as a member of the toll-like receptor family[39]. We returned to bench research to resolve this query. To validate the specific pathway of choice, pharmacological and genetic methods are necessary and not all knockout mice are available. Indirect opioid receptor mechanisms could be tested using mu knock-out mice such as B6.129S2-*Oprm1*^{tm1kff}/J, and non-opioid receptor mechanisms can be measured with B6(Cg)-*Tlr4*$^{tm1.2Karpe}$/J).

5. Upregulation of the OGF-OGFr Axis – Increased Cell Replication

A warning should be posted on these nearly unanimous positive outcomes: "More is not better". The science did not prove the hypothesis that mice with human cancer cells that were inoculated with higher doses of naltrexone should have even smaller tumors than mice inoculated with tumor cells and receiving low doses of naltrexone. The assumption is wrong that if the low dose inhibited tumors, more naltrexone would be even more efficacious. Anecdotally, it is disturbing that as scientists we have a reluctance to understand that the duration of opioid receptor blockade alters response, and that clinicians and/or scientists should not supply more than 4 or 4.5 mg naltrexone daily. Harm may occur with higher doses.

5.1. UPREGULATION OF THE OGF-OGFR AXIS – DIABETES COMPLICATIONS

On the flip side of this is the use of high doses of naltrexone to invoke a continuous blockade of OGFr. Some pathologies led to an upregulation of both peptide and receptor. Diabetes is one disease in which OGF levels in plasma and tissue are elevated in humans[40,41]. This OGF-OGFr axis dysfunction led the way for a number of studies in our laboratory on treatments that continuously blocked the interaction between OGF and OGFr over a 24-hour period between injections or treatments. Diabetic individuals

have many complications over the course of their disease[42]. Those that affect vision are the most compromising. Diabetic retinopathy, along with keratosis, and dry eye are among the disorders that impact the quality of life for diabetes[43]. The upregulation of the OGF-OGFr axis pumps out OGF, raising plasma levels in diabetes, and ultimately causing dysregulation in cell replication.

5.2. PRECLINICAL DIABETES COMPLICATIONS – CORNEAL EPITHELIALIZATION

Corneal abrasions are delayed in the hyperglycemic state leading to ulcers and erosions, and possibly visual loss[43,44]. Knowing that OGF is elevated in this disorder, the laboratory designed early experiments to evaluate cell replication of the corneal surface[45-47]. *In vivo* and *in vitro* studies demonstrated that replication in the corneal epithelium was modulated by the OGF-OGFr pathway because receptor blockade by naltrexone enhanced the outgrowth and organization in culture[46] and accelerated epithelialization in wounded rats[47,48]. Using human donor corneal tissue placed in culture, as well as normal human corneal epithelial cells in culture, OGF was shown to inhibit replication and the addition of NTX accelerated cell proliferation[46]. Diabetic corneal epithelium that was abraded from rats, mice, and rabbits exhibited slower turn-over than ocular epithelium in normal animals[49-52]. In a variety of diabetic species that were models of both type 1 and type 2 diabetes topical application of naltrexone facilitated reepithelialization. The addition of insulin to control hyperglycemia in type 1 diabetic rats and rabbits did not interfere with the efficacy of naltrexone, but there was no evidence of synergy. Intraperitoneal administration or topical application of naltrexone blocked the OGFr and restored cell proliferation.

Prior to clinical trials, the FDA required that a secondary non-rodent model was shown to have the same level of efficacy from naltrexone. New Zealand white rabbits were induced with alloxan to establish type 1 diabetes[50]. After several weeks of hyperglycemia, a dosage of 10^{-4} M naltrexone or vehicle was topically administered 4 times daily for 7 days. The corneas of one eye per rabbit had previously been abraded. Both uncontrolled and insulin-controlled type 1 diabetic rabbits, as well as non-diabetic normals were studied. Corneal epithelialization was delayed in the type 1 diabetic rabbits. Type 1 diabetic rabbits treated with naltrexone had 64-82% smaller wounds in just 3 days than either normal rabbits or diabetic animals receiving vehicle. Insulin-controlled diabetic rabbits had rates of re-epithelialization that were slower than those receiving naltrexone but faster than that recorded for non-insulin-controlled diabetic rabbits, suggesting that naltrexone acts independently of insulin, but that insulin control of hyperglycemia contributes to some biological activities[50].

5.3. DIABETES COMPLICATIONS – DRY EYE DISEASE

During these investigations one of our clinical collaborators commented that diabetes is often accompanied by dry eye. The pathophysiology of dry eye is not well established but may, in part, involve nerve stimulation that is regulated by the OGF-OGFr pathway, as well as contributions from

the limbus, conjunctiva, and epithelial lacrimal glands[53]. In preclinical studies[55-58], dry eye and corneal surface insensitivity were detected in diabetic rats within weeks of inducing hyperglycemia[54,55,58]. A number of controlled and randomized preclinical studies with diabetic rats utilized non-invasive parameters including tonopen, pachymeter, slit lap, and retinal camera to examine the corneal surface. Dry eye was monitored by the Schirmer test which measures wetting capability over a standard time. In type 1 diabetic rats, a solution of 10^{-5} M naltrexone administered four times daily for 1 or 5 days re-established tear volumes. The effect was recorded within 1 hr of administration of the first drop. The restoration of dry eye lasted about 3 days following 5 days of treatment, whereas the restitution of corneal sensation lasted up to 7 days[54]. In fact, topical application of insulin had no effect on dry eye, but did restore some level of corneal sensation. Restitution of corneal sensitivity lasted for 4-7 days following 5 days (q.i.d) of naltrexone application[54]. Because type 2 diabetes is more prevalent in the human population than type 1 and affects nearly 85% of individuals with diabetes, we translated our studies to the *db/db* mouse, a model of type 2 diabetes[56]. Naltrexone at dosages of 5 x 10^{-5} M was effective at restoring dry eye in the mice.

Our studies observing the dysregulation of the OGF-OGFr axis in diabetes and the efficacy of NTX as a treatment led to investigations on whether naltrexone treatment could *prevent, not just treat,* the dysregulation[57,58]. Animals were treated topically with naltrexone drops concomitantly with becoming hyperglycemic. Studies on male and female Sprague-Dawley rats revealed that the timing is slightly different between male and female diabetic rats. Females developed ocular defects, such as dry eye and corneal sensitivity a few days earlier that male diabetic rats[58]. The magnitude of ocular surface defects was nearly comparable. Insulin therapy to regulate hyperglycemia was independent of the OGF-OGFr axis as insulin altered blood glucose but had no effect on dry eye and/or corneal epithelial sensitivity.

The observations on efficacy were moved back to the clinic and a small phase 1 trial was designed to measure the ability to tolerate the application of naltrexone drops in volunteers[59]. Twenty subjects were recruited and self-administered NTX or placebo over a 24 hr period of time. No significant adverse events were reported supporting further human studies. A phase 2 clinical trial was conducted utilizing diabetic persons with dry eye. The arms of the trial were underpowered such that no sufficient efficacy data was obtained. Anecdotal statements of less dry eye were made. No adverse events occurred.

5.4. PERIPHERAL EPITHEALIZATION – DIABETIC FOOT ULCERS

The current topic of translational research involves enhancement of slow-healing full-thickness cutaneous wounds[60-64]. Intellectual property has been filed and issued for the use of a formulation containing naltrexone for treatment of cutaneous wounds, particularly those related to diabetic foot ulcers. Preclinical studies in rats, mice, and swine have determined efficacy and/or safety in all 3 models. Several dosages of proprietary formulations restored the timing of wound closure, enhanced the quality of epithelium

that covered the wound by strengthening it, and increased angiogenesis in surrounding tissues[61,62]. Safety studies that extended to mini-pigs as directed by the FDA were continued with proprietary formulations[64]. Because of the vast amount of information related to the safety of naltrexone, approved use of the drug to combat addictions, and our phase 1-2 clinical trials for dry eye, we are proposing to move directly into Phase 2 clinical trials with another translational application of blockade of the OGF-OGFr pathway.

6. Conclusion

Our translational research has led the team onto pathways that were academically unexpected. Our work in the last 4 decades on the OGF-OGFr axis has been "bench to bedside" as well as 'bedside to bench' and back to bedside. The team has several patent portfolios with issued international and national patents, 4 start-up companies, and a number of grants. Returning to focus on "bench" research, the team has explored the proteomics of diabetic dry eye in male and female rats in order to uncover specific targeted proteins that are dysregulated by diabetes. Because the restoration of dry eye and even epithelial wounds occurs in a relatively short period time (i.e., days), major morphological alterations are most likely not involved. What may be involved are the regulatory proteins that influence secretion of factors, fluids, or other particles that resolve the defects. Nonetheless, the OGF-OGFr axis is powerful, involved in numerous pathologies, but can be modulated easily. Unanswered questions remain. Could there be a formulation of naltrexone that would be safe and effective as a prophylactic treatment for pre-diabetes or diabetes? Would screening for levels of opioid growth factor in the serum provide an early signal for the onset of autoimmune disorders? These and other manipulations of the OGF-OGFr axis warrant further investigation.

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Author contributions

ISZ: conceptualization, data interpretation, funding acquisition, writing – review and editing. PJM: conceptualization, funding acquisition, data interpretation and confirmation of authenticity, project administration and supervision, writing – original draft, review and editing.

Data availability

The data sets used and/or analyzed for the current study are available upon reasonable request from the corresponding author.

Ethics statement

All animal procedures were approved by the Animal Care and Use Committee at Penn State University College of Medicine (Hershey) and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Conflict of Interest

ISZ and PJM have intellectual property owned by Penn State Research Foundation that involves

naltrexone treatment of the ocular surface for treatment of dry eye and separate intellectual property on use of naltrexone for treatment of slow healing cutaneous wounds. ISZ and PJM receive no financial compensation or royalties.

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